

AD \_\_\_\_\_

Award Number: DAMD17-00-1-0048

TITLE: Structure/Function Studies of the Androgen Receptor DNA-Binding Region

PRINCIPAL INVESTIGATOR: Fraydoon Rastinejad, Ph.D.

CONTRACTING ORGANIZATION: University of Virginia  
Charlottesville, Virginia 22904

REPORT DATE: April 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20030306 134

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY (Leave blank)</b>		<b>2. REPORT DATE</b> April 2002	<b>3. REPORT TYPE AND DATES COVERED</b> Annual (1 Apr 01 - 31 Mar 02)	
<b>4. TITLE AND SUBTITLE</b> Structure/Function Studies of the Androgen Receptor DNA-Binding Region			<b>5. FUNDING NUMBERS</b> DAMD17-00-1-0048	
<b>6. AUTHOR(S)</b> Fraydoon Rastinejad, Ph.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> University of Virginia Charlottesville, Virginia 22904  E-Mail: fr9c@virginia.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for public release; distribution unlimited.			<b>12b. DISTRIBUTION CODE</b>	
<b>13. ABSTRACT (Maximum 200 Words)</b>  In this annual report we discuss the progress over the past 12 months towards characterizing the structure-function relationship in the androgen receptor (AR) DNA-binding domain (DBD). We have over-produced large quantities of the AR-DBD using E. coli expression systems, and obtained pure preparations of DNA targets of various size and composition, all of which incorporate the AR binding site. We continue to work towards obtaining the highest resolution X-ray diffraction data from crystals of the AR-DBD/DNA complex and have been optimizing our crystalline preparations. In addition, we have made progress toward characterizing previously unknown functions of the AR-DBD. We report in two manuscripts completed in the previous 12 months (included in the appendix) our findings that the DBD acts as the nuclear export signal for AR and related nuclear receptors, and that this export is dependent on Calreticuling and calcium.				
<b>14. SUBJECT TERMS</b> androgen receptor, x-ray crystallography, DNA-Protein			<b>15. NUMBER OF PAGES</b> 29	
			<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

## Table of Contents

Cover.....	
SF 298.....	2
Introduction.....	4
body.....	4-5
Key Research Accomplishments.....	6
Reportable Outcomes.....	7
Conclusions.....	7
References.....	---
Appendices.....	2 manuscripts

## Structure-Function Studies of Androgen Receptor DNA Binding Region

### INTRODUCTION

The androgen receptor (AR) is a member of the nuclear hormone receptor superfamily, a class of ligand-activated gene-regulatory factors. Nuclear receptors, including AR, regulate gene expression through specific DNA binding sites known as hormone response elements. A specific and conserved polypeptide region, known as the DNA-binding domain (DBD), is generally able to impart the receptor's entire DNA-binding specificity and selectivity. The AR is a good drug target for current and future anti-prostate cancer therapy, because it is responsible for the growth and differentiation of the normal and cancerous cells of the prostate. To date, the ligand binding domain has been the major target of drug development. Our stated objectives are to carry out studies to determine the structure and function of the AR DBD, in order to uncover this region's potential as a future drug target.

### Body:

This past year has seen our progress with regard to our SOW Task2. Specifically, we have identified the role for the Androgen receptor (AR) DNA-binding and hinge regions and examined what functions are associated with these regions of nuclear receptors.

(Appendix-1, Black et al., 2001) contains our first set of findings. We set out to identify nuclear export signals (NES) in the androgen receptor (AR), glucocorticoid receptor (GR) and other related receptors and to characterize their functions related to their nuclear export pathway. We found that the 69 amino acid DNA binding domain (DBD) of these receptors, which is unrelated to any known NES, is necessary and sufficient for export.



Mutational analysis revealed that a 15 amino acid sequence between the two zinc binding loops in the DBD confers nuclear export to a green-fluorescence protein construct, and alanine-scanning mutagenesis was used to identify the residues within this sequence that are critical for export. The DBD is highly related (41%-88% identity) in steroid, nonsteroid, and orphan nuclear receptors, and we found that the DBDs from ten different nuclear receptors all function as export signals. Our findings guided us to propose that NLS-mediated import and DBD-mediated export define a shuttling cycle that integrates the compartmentalization and activity of nuclear receptors.

(Appendix-2, Holaska et al, 2002) contains our second paper which describes how we have recently characterized a pathway for nuclear export of the nuclear receptors in mammalian cells. This pathway involves the  $\text{Ca}(2+)$ -binding protein calreticulin (CRT), which directly contacts the DNA binding domain (DBD) of AR, and related receptors and allows their transport from the nucleus to cytoplasm. We examined a role of  $\text{Ca}(2+)$  in CRT-dependent export of these receptors. Removing  $\text{Ca}(2+)$  from CRT inhibits the stimulation of the nuclear export, an effect due to the failure of CRT to bind the DBD of these receptors. These effects are reversible, when there is a restoration of  $\text{Ca}(2+)$ . Depletion of intracellular  $\text{Ca}(2+)$  inhibits receptor export in intact cells under conditions that do not inhibit other nuclear transport pathways. We also found that the Ran GTPase is not required for GR export. It appears that the nuclear export pathway used by steroid hormone receptors are most likely distinct from the Crm1 pathway. Our data has been leading us to consider whether signaling events that increase  $\text{Ca}(2+)$  could positively regulate CRT and inhibit GR function through nuclear export.

Key Research Accomplishments Over the Previous Year

The above section and the related Appendices summarize our work with regard to characterizing the function of the AR DBD and Hinge regions. One remaining objective is to work towards the structure of

the AR-DBD with DNA. We have carried out a number of gel mobility shift assays to uncover the requirements in terms of overall length, sequence of half-sites and flanking regions that enhance the interactions of the AR-DBD as a homodimer. In the previous report we stated that a number of these DNA duplexes were in used to generate co-crystals suitable for X-ray diffraction. We also described how we had made a series of DNA-binding expression constructs which allowed us to generate pure AR-DBD protein isolated from *E. coli*. These reagents were used to generate single crystals for X-ray diffraction studies. We have worked over the past period to optimize the quality of these crystals, and to generate the highest resolution diffraction data possible, and also phasing information to generate the structure of interest. For phasing, we are designing DNA duplexes in which specific thymidine bases are substituted with 5-iodo-uridines, with the goal to generate heavy atom derivatives suitable for phasing the structure via MIR methods and difference Pattersons. These studies are all underway, and we expect the crystal structure of the AR-DBD homodimer bound to DNA will be solved and published over the next 12 months.

A second objective is to better understand the functions of the AR-DBD. We have made considerable inroads in the past year towards this goal. In collaboration with another laboratory at the University of Virginia, we reported our findings that the AR DBD, as well as the DBDs of many other nuclear receptors, function as their nuclear export signals (see publication in Appendix by Black et al., 2001). In a follow-up study, we also identified how calcium regulates the nuclear export activity of AR and other receptors, via Calreticulin (see manuscript in Appendix by Holaska et al., 2002).

### Reportable Outcomes

A) Five E. coli over-expression clones for producing AR-DBD.

B) Publications and Manuscripts Pending Publication:

- i) Black, B.E., Holaska, J.M, Rastinejad, F., Paschal, B.M.  
DNA binding domains in diverse nuclear receptors function as nuclear export signals. *Current Biology*, 11: 1749-1758 (2001).
- ii) Holaska, J.M., Black, B.E., Rastinejad, F., Paschal, B.M.  
Ca<sup>2+</sup> regulates the nuclear export activity of calreticulin.  
Submitted *Mol. Cell. Biol.* (2002).

### Conclusions:

The past year has seen our continued work towards obtaining more information about the function of the AR DNA-binding domain and hinge region. Specifically, we have identified a previously unknown function of the AR-DBD in acting as the nuclear export signal. We have also made further inroads towards obtaining a crystal structure of the AR DBD in complex with its DNA target.

### Appendix 1 and 2:

A Publication and a Manuscript Submitted for Publication:

Black, B.E., Holaska, J.M, Rastinejad, F., Paschal, B.M.  
DNA binding domains in diverse nuclear receptors function as nuclear export signals. *Current Biology*, 11: 1749-1758 (2001).

Holaska, J.M., Black, B.E., Rastinejad, F., Paschal, B.M.  
Ca<sup>2+</sup> regulates the nuclear export activity of calreticulin.  
Submitted *Mol. Cell. Biol.* (2002).

# DNA binding domains in diverse nuclear receptors function as nuclear export signals

Ben E. Black<sup>\*†‡</sup>, James M. Holaska<sup>\*</sup>, Fraydoon Rastinejad<sup>†‡§</sup>  
and Bryce M. Paschal<sup>\*†‡</sup>

**Background:** The nuclear receptor superfamily of transcription factors directs gene expression through DNA sequence-specific interactions with target genes. Nuclear import of these receptors involves recognition of a nuclear localization signal (NLS) by importins, which mediate translocation into the nucleus. Nuclear receptors lack a leucine-rich nuclear export signal (NES), and export is insensitive to leptomycin B, indicating that nuclear export is not mediated by Crm1.

**Results:** We set out to define the NES in the glucocorticoid receptor (GR) and to characterize the export pathway. We found that the 69 amino acid DNA binding domain (DBD) of GR, which is unrelated to any known NES, is necessary and sufficient for export. Mutational analysis revealed that a 15 amino acid sequence between the two zinc binding loops in the GR-DBD confers nuclear export to a GFP reporter protein, and alanine-scanning mutagenesis was used to identify the residues within this sequence that are critical for export. The DBD is highly related (41%–88% identity) in steroid, nonsteroid, and orphan nuclear receptors, and we found that the DBDs from ten different nuclear receptors all function as export signals. DBD-dependent nuclear export is saturable, and prolonged nuclear localization of the GR increases its transcriptional activity.

**Conclusions:** Multiple members of the nuclear receptor superfamily use a common pathway to exit the nucleus. We propose that NLS-mediated import and DBD-mediated export define a shuttling cycle that integrates the compartmentalization and activity of nuclear receptors.

## Background

Nuclear receptors contain a structurally conserved DBD that contacts the respective DNA response element within enhancers of target genes [1–3]. Ligand-occupied nuclear receptors interact with transcriptional coactivators, whereas ligand-free nuclear receptors interact with transcriptional repressors. Ligand binding has, in addition, a profound influence on the subcellular distribution of certain nuclear receptors [4]. Glucocorticoid binding to GR induces receptor translocation from the cytoplasm to the nucleus [5], and androgen induces translocation of the androgen receptor (AR) to the nucleus as well [6]. Nuclear import occurs because ligand binding to nuclear receptors releases chaperones and exposes one or more NLSs. GR contains two ligand-regulated NLSs, one of which is located in the hinge region adjacent to the DBD [5]. The NLS is, in turn, recognized by nuclear import factors that facilitate translocation of the nuclear receptor through the nuclear pore complex and delivery to the nucleoplasm [7, 8].

Certain nuclear receptors have also been shown to undergo export from the nucleus. Hormone withdrawal from

cells induces the cytoplasmic accumulation of GR and AR [6, 9]. Moreover, in the presence of ligand where nuclear receptors appear constitutively nuclear, GR, AR, thyroid hormone receptor (TR), and the progesterone receptor (PR) are rapidly shuttling between the nucleus and cytoplasm [6, 10–13]. Because the sizes of these and other nuclear receptors (43–90 kDa) exceeds the molecular weight cutoff for simple diffusion through the nuclear pore, it has been inferred that nuclear export of nuclear receptors is a facilitated process. The balance of nuclear import and export could be an important mechanism for regulating the transactivation potential of nuclear receptors. Indeed, selective nuclear localization of factors involved in signal transduction and transcription has emerged as an important regulatory step in several biological pathways [14].

The best-characterized nuclear export pathway uses Crm1 as a receptor for proteins that contain a leucine-rich NES. Nuclear receptors such as GR, AR, TR, and PR do not, however, contain a leucine-rich NES, suggesting that these proteins are not transport substrates for Crm1. This view is corroborated by the observation that the Crm1-

Addresses: <sup>\*</sup>Center for Cell Signaling, <sup>†</sup>Department of Biochemistry and Molecular Genetics, <sup>‡</sup>Cell and Molecular Biology Program, and <sup>§</sup>Department of Pharmacology, University of Virginia, Charlottesville, Virginia 22908, USA.

Correspondence: Bryce M. Paschal  
E-mail: paschal@virginia.edu

Received: 13 August 2001  
Revised: 11 September 2001  
Accepted: 27 September 2001

Published: 13 November 2001

Current Biology 2001, 11:1749–1758

0960-9822/01/\$ – see front matter  
© 2001 Elsevier Science Ltd. All rights reserved.

specific inhibitor leptomycin B does not inhibit nuclear export of GR, PR, AR, or TR in mammalian cells [9, 13, 15–17]. Recent insight into how nuclear receptors may exit the nucleus came from a biochemical screen for nuclear export factors present in extracts prepared from mammalian cells [17]. The calcium binding protein calreticulin (CRT) was identified as a factor that stimulates nuclear export of proteins that contain a leucine-rich NES [17]. The previous link between CRT and downregulation of GR, AR, all-trans retinoic acid receptor (RAR), and vitamin D receptor (VDR) transcriptional activity [18–20] suggested a potential role in the export of nuclear receptors as well, despite the absence of a leucine-rich NES in these proteins. Recombinant CRT stimulates nuclear export of GR in permeabilized cell assays, and GR export, which is deficient in *crt*<sup>-/-</sup> cells, can be complemented by back-transfecting CRT [17].

Here we characterize the signal for nuclear export of GR, which is contained within its DBD. Moreover, the DBD functions as the export signal in multiple steroid, nonsteroid, and orphan nuclear receptor family members. The DBD export pathway is saturable, indicating that nuclear receptors compete for a limited number of soluble transport factors, NPC binding sites, or both. Overexpression of the VDR DBD inhibits GR export and results in an elevated transcriptional response of GR to dexamethasone (Dex). Thus, the DBD export pathway contributes to the regulation of GR and probably other nuclear receptors through a nuclear transport-based mechanism.

## Results

### The DBD of GR contains the NES

The DBD of GR can be incorporated into a green fluorescent protein (GFP) reporter and can function as an NES when expressed in cultured cells [17]. The conserved sequence and structure of the DBD in different nuclear receptors (Figure 1a,b) led us to hypothesize that the DBD is widely used as an export signal. To address this hypothesis, we first mapped the molecular determinants of the 69 amino acid GR DBD that are necessary for nuclear export by using a GFP reporter that undergoes ligand-dependent nuclear import (Figure 1c). Following ligand removal from the cell culture media, the distribution of the GFP reporter was recorded at 0, 2, 4, and 6 hr by fluorescence microscopy. We found that cysteine to alanine mutations that disrupt the zinc binding loops and abolish DNA binding only slightly inhibited nuclear export of the GFP reporter (C424A and C463A; Figure 2). This suggested that nuclear export might instead rely on the DNA recognition helix that is situated between the two zinc binding loops. Indeed, a GFP reporter that contained only 15 amino acids of the GR DBD (442–456) underwent translocation from the nucleus to the cytoplasm. Although the zinc binding loops of nuclear receptors do not contain information that is critical for export

specified by the DBD, there may be a contribution to the DBD structure that augments recognition of the signal by the export machinery.

Alanine-scanning mutagenesis of the DNA recognition helix between the loops revealed that a pair of phenylalanines is critical for the export function of the DBD (FF→AA; Figure 2). Mutating other pairs of amino acids between the zinc fingers (KR→AA, VE→AA, and YL→AA) impaired the nuclear export function of the GR DBD to a lesser degree (2 and 4 hr time points). In contrast, other mutations (KV→AA, GQ→AA, and HN→AA) had no effect on the export activity of the GR DBD. Our data show that amino acids between the two zinc binding loops, which include part of the DNA recognition helix, contain the NES of GR.

### Steroid, nonsteroid, and orphan receptors use a common export pathway

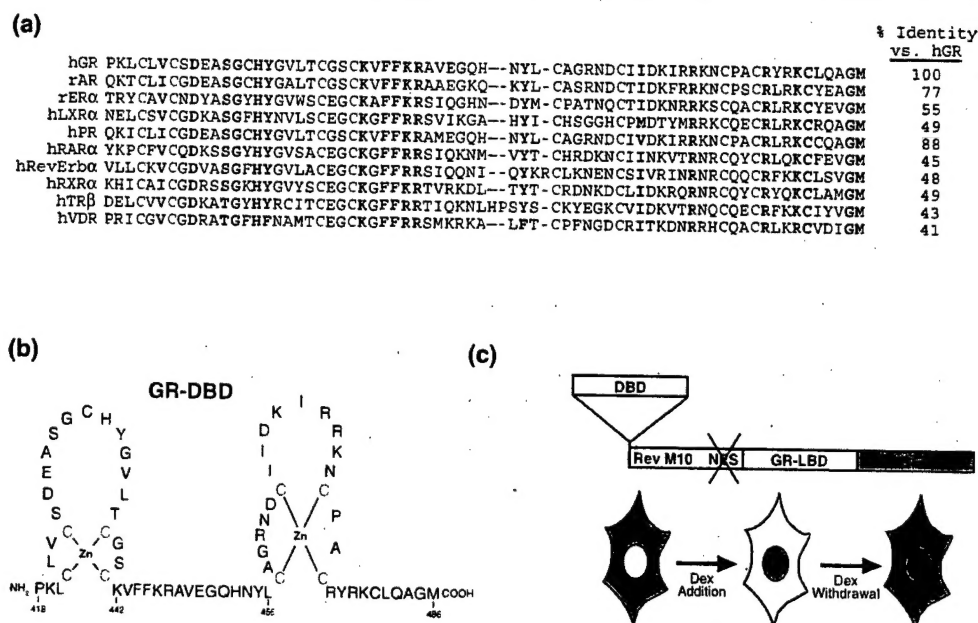
Our finding that the GR DBD contains the NES appeared significant in light of the structural conservation of the DBD among nuclear receptors. The GR DBD is at least 40% identical to the sequences of DBDs from nine different nuclear receptors included in our analysis (Figure 1a). Moreover, every member of the nuclear receptor superfamily that has been identified to date contains a pair of phenylalanines that are located at the same position relative to phenylalanines within the GR DBD. This led us to hypothesize that the DBDs from diverse nuclear receptors can function as an NES. We tested this hypothesis by analyzing the export activity of DBDs from steroid, nonsteroid, and orphan nuclear receptors. We found that nuclear export of the GFP reporter was conferred by the DBD derived from AR, estrogen receptor (ER), liver X receptor (LXR), PR, RAR, RevErb, retinoid X receptor (RXR), TR, and VDR (Figure 3). Nuclear export was not conferred by the DBD derived from GATA1, a transcription factor that, like the nuclear receptors, contains two zinc binding loops in its DBD [21]. GATA1 is not, however, a member of the nuclear receptor superfamily based on sequence or structure. Thus, our data identify the DBD of nuclear receptors as a new type of NES. The DBD does not contain a leucine-rich NES that is recognized by the export receptor Crm1, which may explain why nuclear export of GR, PR, TR, and AR are insensitive to leptomycin B [9, 13, 15–17].

### Mutations in the DBD block nucleocytoplasmic shuttling of nuclear receptors

Our experiments demonstrated that the DBD is sufficient to mediate nuclear export in the context of the GFP reporter. To determine whether the DBD is necessary for export in the context of the receptor, we examined nucleocytoplasmic shuttling of full-length, wild-type (wt) GR, AR, and RAR, and of mutant forms of these receptors that were predicted to be deficient for export. We designed a modified-shuttling assay that, in a heterokaryon



Figure 1



Conserved structure of the DBD and assay for nuclear export activity. (a) Alignment of DBDs from nuclear receptors used in this study and the percent identity to the DBD of human GR. Highly conserved residues (bold) including the cysteines that coordinate zinc binding (green) and the pair of phenylalanines that are present in the DNA recognition helix of all nuclear receptors (red) are indicated. (b) Diagram of the GR DBD showing the position of the pair of phenylalanines relative to the zinc binding loops. (c) Features of the

GFP reporter and two-step assay for nuclear export in vivo. The vector encodes a mutant form of Rev (M10) that contains a nonfunctional NES fused to the ligand binding domain (LBD) of GR and GFP. Dex induces nuclear import of the GFP reporter, which remains nuclear after Dex withdrawal because the reporter lacks a functional NES. In-frame fusion of a DBD to the N terminus of the GFP reporter restores nuclear export activity.

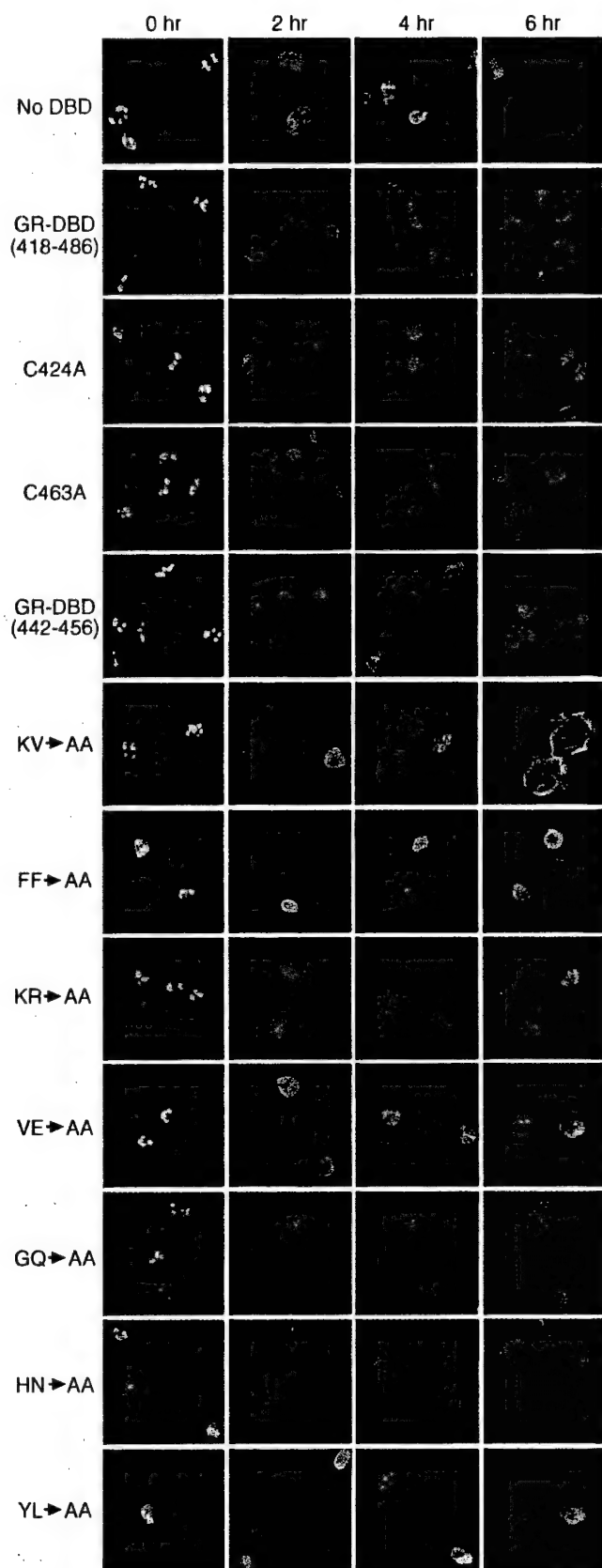
fusion, scores nuclear export from a donor nucleus and nuclear import into an acceptor nucleus. Prior to fusion (Figure 4a, upper panels), the donor cells (human) were transfected with GFP fusions of the receptors and treated with the appropriate ligand to induce nuclear import. The acceptor cells (mouse) were labeled with a CellTracker dye that, following its uptake, is converted to a membrane impermeant form in the cytoplasm. After fusion (Figure 4a, lower panels), the cytoplasm of the heterokaryon fluoresces red, and the GFP fusion (green) containing full-length GR equilibrates between the human and mouse nuclei because it undergoes shuttling, as previously shown [11]. The GFP reporter used in prior experiments to characterize the DBD (Figure 1c) undergoes nuclear import, but fails to undergo export and equilibrate between nuclei in the heterokaryon because it lacks a functional NES. Nuclear export of the GFP reporter was restored by the GR DBD, resulting in equilibration of the reporter between nuclei in the heterokaryon fusion (Figure 4b).

The GFP reporter used in the experiments described above contains the ligand binding domain of GR to direct Dex-dependent import. We used another GFP reporter, which lacked any GR sequence, to formally test for the sufficiency of the DBD export signal. The GFP reporter

used contains the SV40 large T antigen NLS to direct constitutive import, but it remains nuclear in the heterokaryon because it does not contain a nuclear export signal (Figure 4c, upper panels). Fusion of the GR DBD to the reporter resulted in nucleocytoplasmic shuttling (Figure 4c, lower panels). Thus, the GR DBD is sufficient to direct nuclear export of the GFP reporter.

We next transfected full-length nuclear receptors (GR, AR, and RAR) containing either a wt DBD or a mutant DBD (FF→AA) into donor cells, treated the cells with ligand, and fused them with acceptor cells labeled with the CellTracker dye. The wt forms of GR, AR, and RAR equilibrated between nuclei in the heterokaryon fusion (Figure 4d), indicating that export of these nuclear receptors occurs even in the presence of ligand. The DBD mutant forms (FF→AA) of GR and AR, however, failed to equilibrate between nuclei in the heterokaryon fusion. Thus, export of GR and AR from the donor nuclei is inhibited by changing only two amino acids within the DBD of each nuclear receptor. Surprisingly, the DBD mutant form of RAR still equilibrated between nuclei in the heterokaryon fusion (Figure 4d). Because the DBD of RAR is sufficient to specify nuclear export of the GFP reporter used in our analysis (Figure 2), we interpret this

Figure 2



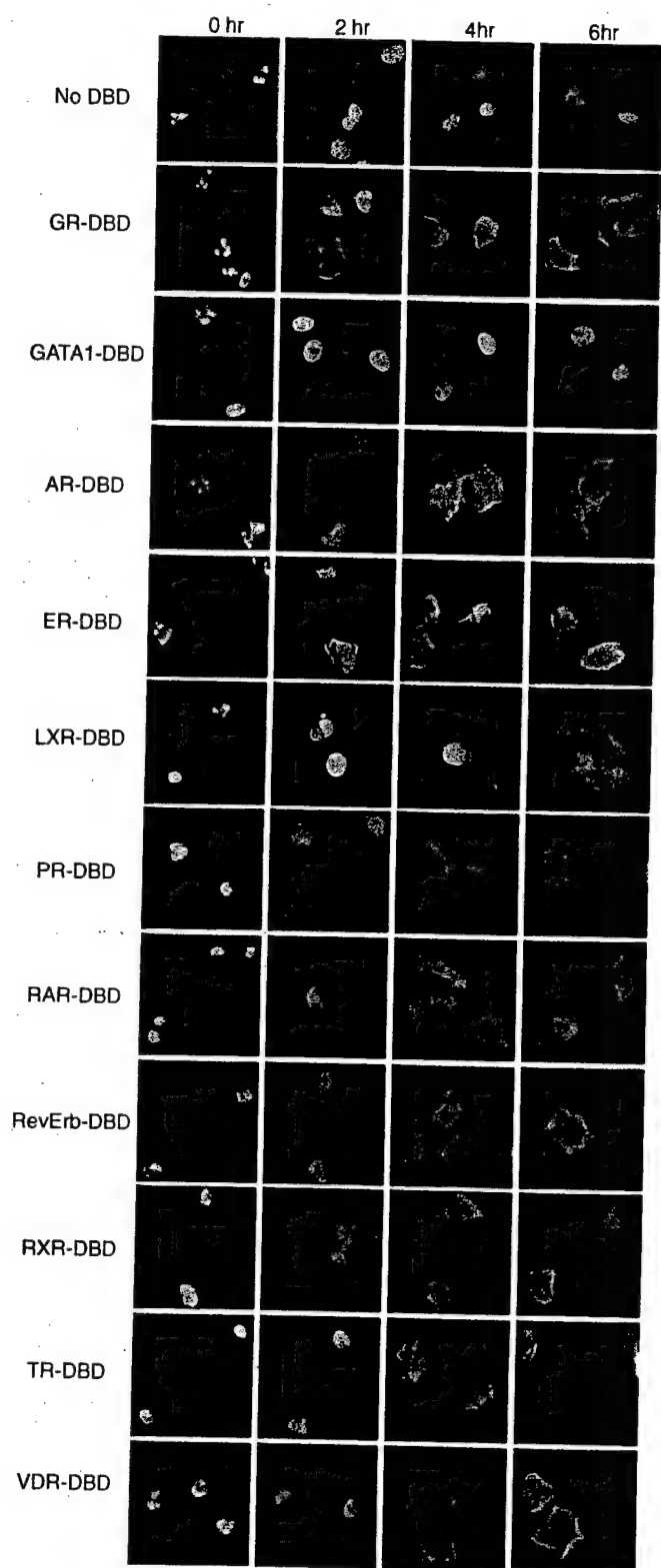
as evidence that RAR export can occur by an alternative, DBD-independent transport pathway. Nuclear export of RXR in nerve cells can be facilitated by its binding partner NGFI-B, an orphan nuclear receptor with three leucine-rich NESs [22]. Nuclear export of the wild-type and mutant forms of RAR, however, still occurs in the presence of leptomycin B (data not shown). Certain nuclear receptors may, therefore, be exported from the nucleus by more than one transport pathway.

#### CRT binding to the GR DBD is required for nuclear export

Our results showing that the GR DBD functions as an export signal together with our previous finding that GR export is defective in *crt*<sup>-/-</sup> cells suggested a molecular mechanism for the transport pathway [17]. This could involve CRT-dependent recognition of the GR DBD in the nucleus and translocation of the complex to the cytoplasm. We reasoned that if CRT binding to the DBD is an obligate step in GR export, then mutations within the DBD that inhibit export might also inhibit the interaction of GR with CRT. We tested this by using binding assays with recombinant CRT and glutathione-S-transferase (GST) fusions of the wt and mutant (FF→AA) forms of the GR DBD. CRT bound to the wt DBD but displayed only background binding to the GR DBD mutant protein (Figure 5a). Crm1 did not bind to the GR DBD, consistent with other data indicating that Crm1 is not the receptor for nuclear export of GR [16, 17].

The net redistribution of GR from the nucleus to the cytoplasm generally requires several hours in ligand-free media; however, the kinetics of export can be enhanced by microinjecting recombinant CRT into cells [17]. We used this approach to examine whether GR containing a wt or mutant DBD would undergo CRT-dependent nuclear export. Cos cells were transfected with GFP fused to full-length GR (wt or FF→AA mutant) and treated with ligand to induce nuclear import of GR. The cells were then injected with CRT, and the distribution of GR was recorded by fluorescence microscopy 30 min postinjection.

The DBD of GR functions as an NES. The GFP reporter engineered with the DBD of GR was transfected into Cos cells and assayed for nuclear export. Images of living cells were recorded by fluorescence microscopy at 0, 2, 4, and 6 hr following Dex withdrawal. The GFP reporter alone (no DBD) remains nuclear during the course of the experiment, while including the GR DBD (residues 418–486) in the GFP reporter confers export by 4 hr. Point mutations in the GR DBD (C424A or C463A) slightly reduced the level of export, evident at 4 hr. The 15 residue sequence (KVFFKRAVEGQHNYL; residues 442–456) that resides between the two zinc binding loops was sufficient for export of the GFP reporter. Alanine-scanning mutagenesis of residue pairs in the DBD revealed that the FF→AA mutation in the DNA recognition helix causes a major defect in nuclear export. The KR→AA, VE→AA, and YL→AA mutations have an intermediate effect on nuclear export, and the KV→AA, GQ→AA, and HN→AA mutations have no effect on nuclear export.

**Figure 3**

The DBDs of diverse nuclear receptors can all function as an NES. The DBDs from hormone, nonhormone, and orphan nuclear receptors were analyzed for nuclear export activity *in vivo* by using the GFP reporter. The GFP reporter alone (no DBD) remains nuclear during the course of the experiment. The DBD from the transcription

We observed that injection of cells with CRT caused the relocalization of wt GR from the nucleus to the cytoplasm (Figure 5b, upper panels). In contrast, CRT injection into cells expressing the GR DBD mutant (FF→AA) did not affect GR distribution (Figure 5b, middle panels). Crm1 injection did not cause wt GR redistribution to the cytoplasm. Thus, a functional export signal within the DBD is necessary for CRT-dependent translocation of GR from the nucleus to the cytoplasm. Recombinant CRT can also mediate nuclear export of GR in a permeabilized cell assay [17], and this can be inhibited by the addition of the DBD from VDR (Figure 5c). The ability of the DBD from VDR to act as a competitive inhibitor of GR export indicates that CRT recognizes similar molecular determinants in the DBDs of different nuclear receptors.

#### Excess DBD blocks GR export and increases transcription

Our finding that the VDR DBD can act as a competitive inhibitor of GR export in permeabilized cells led us to examine whether overexpression of the VDR DBD fused to BFP would compete with the GR export pathway in living cells. Because the DBD of VDR also contains an NLS [23], it undergoes export and import but is concentrated in the nucleus at steady state. Expression of BFP alone did not affect nuclear export mediated by the GR-DBD examined at 0 and 5 hr (Figure 6, upper panels). In contrast, expression of BFP-VDR DBD inhibited GR-DBD export and resulted in nuclear accumulation of GR-DBD (Figure 6, middle panels). This effect required a functional DBD in VDR since mutating the conserved pair of phenylalanines in the DBD reversed the inhibition (Figure 6, lower panels). Our data support the hypothesis that a common pathway is used for nuclear export of both steroid (GR) and nonsteroid (VDR) nuclear receptors.

Inhibiting DBD-dependent export *in vivo* restricts GR to the nucleus, thereby blocking the nucleocytoplasmic shuttling cycle. This allowed us to examine whether blocking nuclear export could augment the GR-dependent transcription by increasing its dwell time in the nucleus. We also considered the possibility that nucleocytoplasmic shuttling could be necessary for the transcriptional activity of GR because, for example, cytoplasmic chaperones are required for ligand binding. GR-dependent transcription can be measured in the presence of excess VDR DBD because VDR does not bind to a GRE [24]. The response elements for GR and VDR differ in sequence, direction, and spacing of the half-sites.

factor GATA1, which is similar in size to the nuclear receptors and contains two zinc binding loops, does not confer nuclear export to the GFP reporter. The DBDs from nine different nuclear receptors (AR, ER, LXR, PR, RAR, RevErb, RXR, TR, and VDR) confer nuclear export activity to the GFP reporter.



Figure 4

The DBD export signal is necessary for hormone receptor shuttling in vivo. **(a)** Heterokaryon shuttling assays were performed with Cos cells transfected with full-length GR fused to GFP (FITC) and NIH 3T3 cells labeled with the dye CellTracker CMTMR (Rhodamine). When cosseeded on coverslips and fused by brief (30 s) incubation in polyethylene glycol (Roche; 50% vol:vol), the Cos and 3T3 cells fuse and fluoresce red. Nucleocytoplasmic shuttling (export and import) of the GFP reporter results in equilibration of green fluorescence between the donor Cos cell nuclei and the acceptor 3T3 cell nuclei within the heterokaryon. Acceptor cell nuclei (white arrowheads) are also distinguished by centromeric foci that stained brightly with DAPI. **(b)** The GR DBD restores shuttling behavior to the GFP reporter that contains a nonfunctional NES. **(c)** The GR DBD confers shuttling activity to a GFP reporter that contains the SV40 large T antigen NLS [38]. The GFP reporter contains the streptavidin gene and assembles into a ~160 kDa tetramer that is too large to escape the nucleus by diffusion. **(d)** The DBD export signal is necessary for nucleocytoplasmic shuttling in the context of full-length hormone receptors. Full-length wt or mutant (FF→AA) GR, AR, and RAR were tested for nucleocytoplasmic shuttling in the presence of 1  $\mu$ M Dex, 10 nM R1881 (synthetic androgen), or 1  $\mu$ M all-trans retinoic acid, respectively. Each wt receptor equilibrates between the nuclei of a heterokaryon. The FF→AA mutation inhibits GR and AR shuttling, whereas the FF→AA mutation has only a slight effect on RAR shuttling. The FF→AA mutation in the GR DBD abolishes DNA binding in vitro, suggesting the mutation does not generate a nuclear retention signal (data not shown).

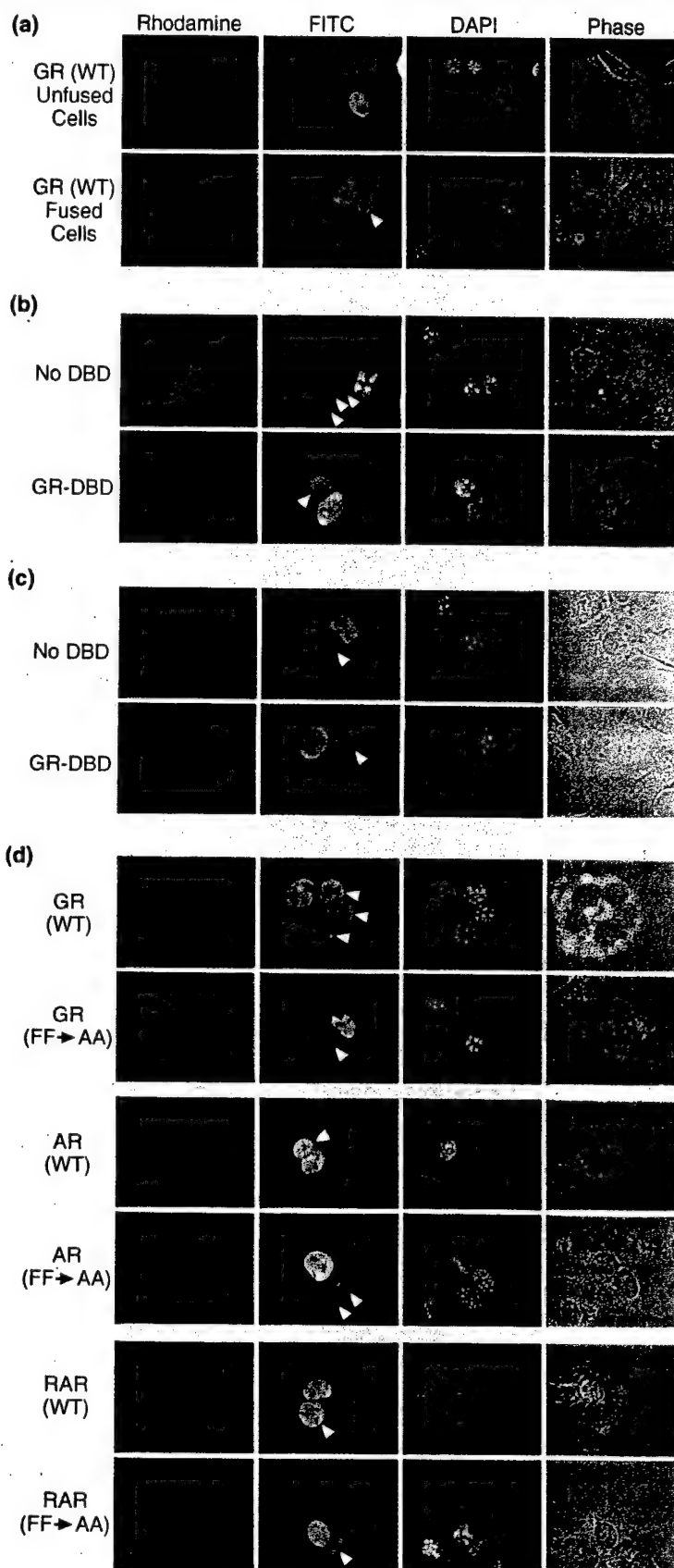
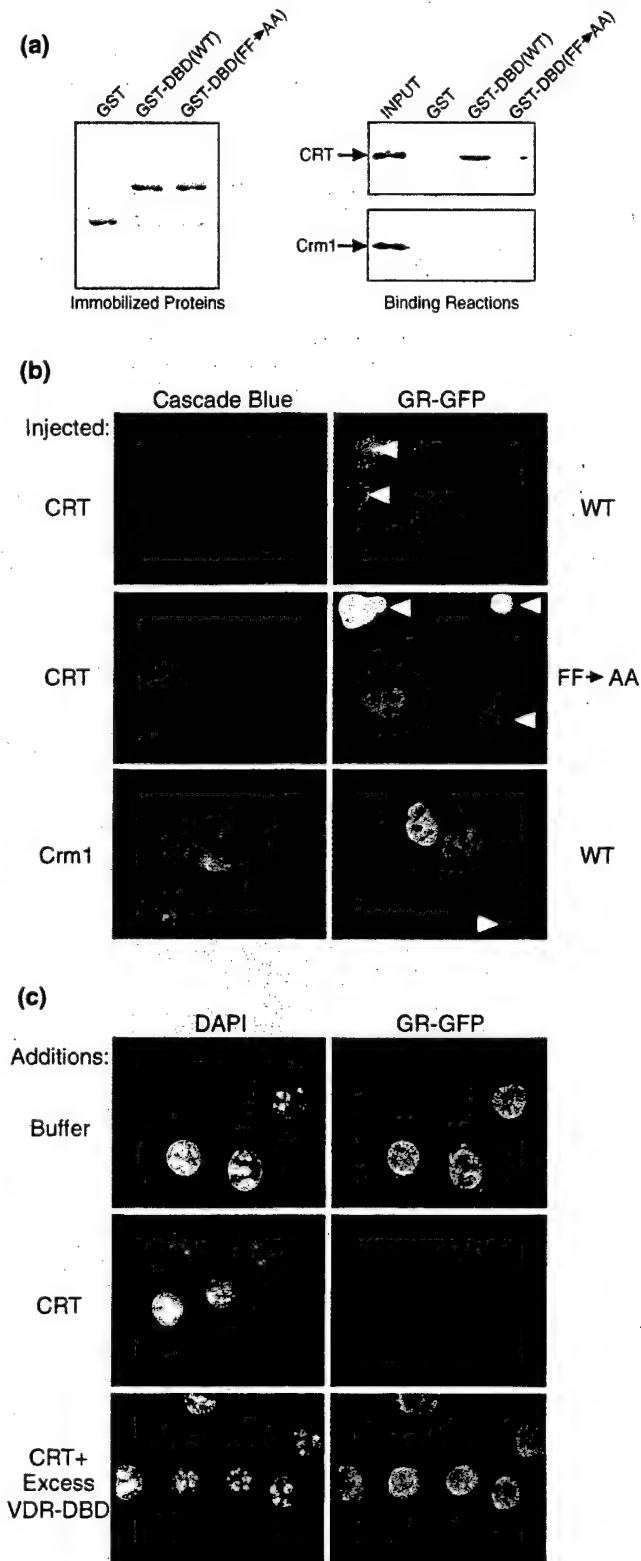


Figure 5



The DBD export signal is necessary for export mediated by CRT. **(a)** Direct binding of CRT to the GR DBD. GST fusion proteins containing the GR DBD (wt or FF→AA) were immobilized on glutathione-Sepharose beads and used for binding assays with recombinant CRT and Crm1. CRT binds to the wt DBD from GR but

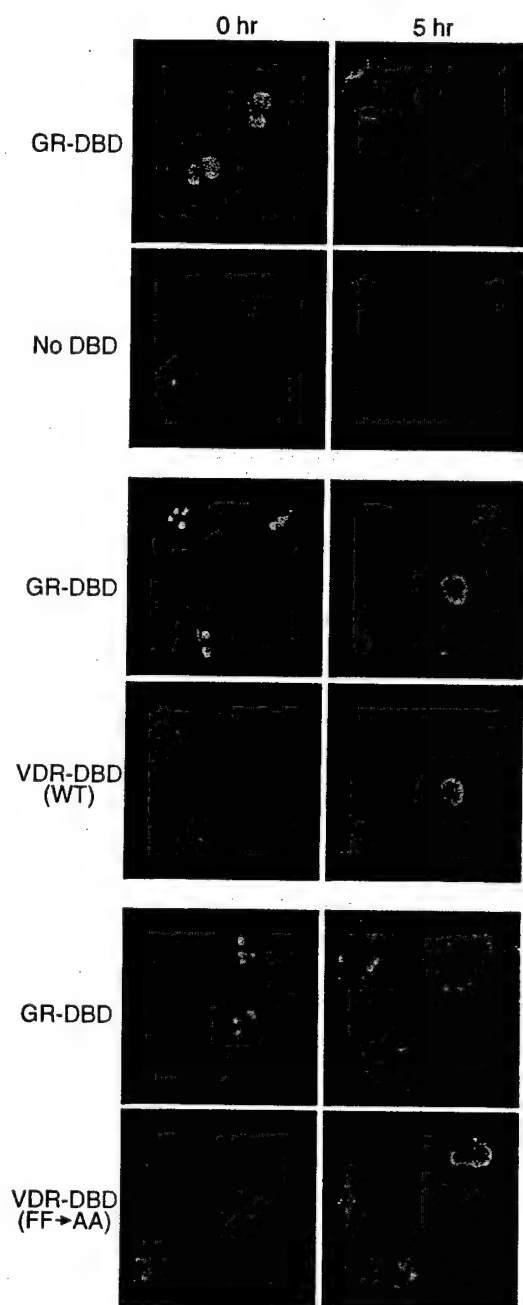
GR-dependent transactivation of a GRE-luciferase reporter was measured in the presence of cotransfected BFP or BFP-VDR DBD as a function of Dex concentration (Figure 7a). Expression of the BFP-VDR DBD increased GRE luciferase activity 2-fold relative to BFP. Thus, inhibiting GR export results in an increase in GR-dependent transcription. The effect was correlated with the amount of BFP-VDR DBD plasmid used for cotransfection (Figure 7b). We also found that a mutant form of the VDR DBD (FF→AA) that has reduced activity as a competitive inhibitor of GR export (Figure 6) increases the transcriptional activity of GR only slightly (Figure 7c). Our data indicate that nuclear export of GR is critical for proper regulation of its transcriptional activity, since blocking GR export results in an elevated response to ligand. Moreover, nuclear export is predicted to be important for regulating the cytoplasmic functions that are emerging for certain nuclear receptors.

### Discussion

In the context of a living cell, GR undergoes rapid exchange between chromatin binding sites and the nucleoplasm [25]. Upon dissociating from chromatin, GR may receive input from signaling pathways that regulate its transcriptional activity, either in the form of posttranslational modifications or cofactor interactions [26]. This could occur in the nucleoplasm, or it could occur during the movement of GR through the cytoplasm during its shuttling cycle. The latter scenario would obviate the requirement for nuclear import of cytoplasmic enzymes, or cofactors, possibly to ensure spatial separation from the nuclear receptors. Because GR, AR, and RAR all shuttle in the presence of their respective ligand, nucleocytoplasmic shuttling is predicted to be a general property of nuclear

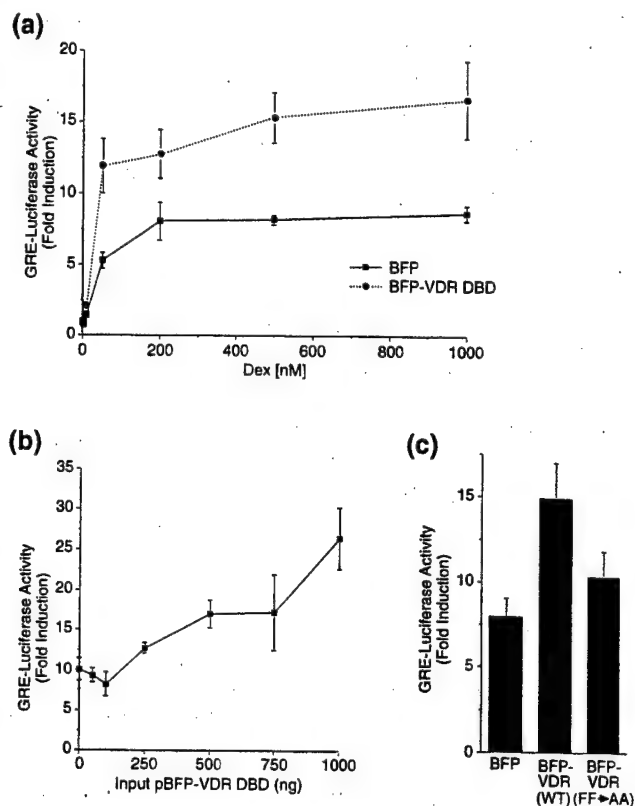
not to the export-defective mutant DBD (FF→AA) from GR. A preparation of recombinant Crm1 that binds the leucine-rich NES [39] does not bind to the DBD from GR. Shown are 5% of the total input proteins (CRT or Crm1). **(b)** CRT stimulates nuclear export of wt but not the export-defective mutant of GR in living cells. Cos cells transfected with GR (wt or FF→AA mutant) fused to GFP were treated with Dex to induce nuclear import. Following Dex withdrawal, the cells were microinjected with either His-tagged CRT or His-tagged Crm1 (1 mg/ml each), and the distribution of GR-GFP was examined after 30 min. A 70 kDa fluorescent dextran (Cascade Blue) was included to mark the injection site and to verify that the nuclear envelope remained intact during the experiment. CRT-dependent export of wt GR was observed in 30/30 cells injected, whereas CRT-dependent export of the mutant GR (FF→AA) was observed in only 2/21 cells injected (three experiments). GR did not undergo nuclear export in uninjected cells (white arrowheads) or Crm1-injected cells in this assay. The latter control is consistent with Crm1-independent export of GR [16, 17]. **(c)** Shared mechanism for nuclear export of GR and VDR. A cell line expressing GR-GFP [35] was permeabilized with digitonin and incubated with recombinant CRT (100 µg/ml) in the absence and presence of the DBD from VDR (120 µg/ml). CRT-dependent export of GR-GFP is blocked by the VDR DBD.

Figure 6



Competition for DBD-mediated export in vivo. The GFP reporter engineered with the DBD of GR was cotransfected with a plasmid encoding BFP alone (no DBD) or BFP fused to the DBD of VDR in Cos cells. Images were captured at 0 and 5 hr following Dex withdrawal. BFP alone did not alter import of the reporter alone (data not shown) or the import and export of the reporter containing the DBD of GR. The DBD of VDR (VDR-DBD [wt]) blocks GR DBD-mediated nuclear export. However, a mutant VDR DBD that has two critical phenylalanines replaced with alanines (VDR-DBD (FF→AA)) does not compete for GR DBD-mediated nuclear export.

Figure 7



Blocking GR export increases ligand-induced gene expression. **(a)** VDR-DBD expression increases GR-mediated gene expression over a wide range of Dex concentration. Endogenous GR activity was examined in NIH3T3 cells using a GRE-luciferase reporter. Cells were cotransfected in 6 well dishes with plasmids encoding a *Renilla*-luciferase reporter (30 ng), GRE-firefly luciferase (370 ng), and either BFP alone or BFP-VDR DBD (1000 ng), and then they were incubated overnight. Cells were lysed 6 hr following the addition of the indicated amount of Dex. **(b)** Increasing input pBFP-VDR DBD increases Dex-induced gene expression. Transfectants were incubated overnight and then treated with Dex (500 nM) for 6 hr prior to lysis. **(c)** Expression of a VDR DBD mutant that fails to compete for GR DBD-mediated nuclear export only slightly increases Dex-induced gene expression. Plasmids encoding either wt or FF→AA mutant versions of BFP-VDR DBD (500 ng each) were transfected and assayed as above. All luciferase assays were normalized for transfection efficiency using *Renilla*-luciferase expression. The values shown represent the fold induction by the addition of Dex and are the averages of triplicate wells plus or minus the standard deviation.

receptors. This would integrate cytoplasmic signaling events with regulation of nuclear transcription [27]. Alternatively, the function of DBD-dependent export may relate to cytoplasmic functions for nuclear receptors that may be transcription-independent [28–30].

Our finding that the DBD of nuclear receptors performs dual targeting functions in the cell is not without precedent. The DBD in Gal4 contains an NLS, and the DBD in Stat1 contains a leucine-rich NES that targets it for export by the Crm1 pathway [31, 32]. Thus, protein do-

mains that bind DNA provide suitable structures for presenting signals to the nuclear transport machinery. Nuclear transport receptor binding to a DBD-containing protein should, in addition, block the interaction of the protein with DNA. In the case of GR and probably other nuclear receptors, this may impart negative regulation on transcription even prior to nuclear export [18–20]. Nuclear export facilitated by DBDs and nuclear import facilitated by ligand-regulated NLSs illustrate how simple nuclear transport-based mechanisms are used to regulate the activity of a superfamily of transcriptional activators and influence gene expression in multiple biological pathways.

## Materials and methods

### Nuclear receptor cDNAs

The DBD from human GR was subcloned from the previously described plasmid pK7-GR-GFP [33]. The GenBank accession numbers corresponding to the other nuclear receptor superfamily members used for the DBD analysis are: rat AR, M23264; rat ER $\alpha$ , NM\_012689; human LXR $\alpha$ , NM\_005693; human PR, NM\_000926; human RAR $\alpha$ , NM\_000964; human RevErbA $\alpha$ , X72631; human RXR $\alpha$ , NM\_002957; human TR $\beta$ , X04707; and human VDR, NM\_000376. Mutations were made using the QuickChange system (Stratagene).

### Nuclear export assays

#### In vivo export assays

The reporter used to test DBD-mediated nuclear export has been described [17] and is derived from the plasmid pXM10 [34]. Cos7 cells were seeded onto coverslips and grown overnight prior to transfection of the reporter vectors using the transfection reagent Eugene 6 (Roche). All BFP-DBD competition experiments used plasmids derived from pEBFP-C (Clontech). The transfectants were grown overnight prior to addition of Dex (1  $\mu$ M) for 45–60 min. Cells were washed five times with serum-free and phenol red-free Dulbecco's modified eagle medium (DMEM) and incubated for the indicated times in phenol red-free DMEM containing 10  $\mu$ g/ml cyclohexamide and 10% charcoal stripped newborn calf or fetal bovine serum. Cells were fixed in formaldehyde (3.7%) and processed for fluorescence microscopy. Digital images were captured by a charge-coupled device camera (Hamamatsu ORCA) mounted on a Nikon Microphot-SA microscope, with Openlab (version 2.0.6) software. Figures were assembled with Adobe Photoshop (version 5.5) and Freehand (version 9). The examples shown are representative of 20–50 cells observed in two or more independent experiments.

#### In vitro export assays

A cell line expressing GR-GFP [35] was treated with 1  $\mu$ M Dex for 45 min. Cells were permeabilized with 0.005% digitonin for 5.5 min. Export reactions were performed at 30°C for 30 min in transport buffer (20 mM Hepes [pH 7.4], 110 mM potassium acetate, 2 mM magnesium acetate, and 1 mM EGTA) supplemented with an ATP-regeneration system, 2 mM dithiothreitol (DTT), and a protease inhibitor cocktail including aprotinin, leupeptin, and pepstatin (each at 1  $\mu$ g/ml). GFP images were captured with the same exposure times.

### Heterokaryon analysis

The modified interspecies heterokaryon analysis was performed as follows. NIH3T3 cells were labeled in tissue culture dishes with 500 nM CellTracker dye, (5 (and 6)-((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (CMTMR; Molecular Probes), according to the manufacturers instructions. Unincorporated dye was removed and cells were trypsinized and coseeded on glass coverslips with Cos7 cells that had been transfected with the indicated plasmids using the transfection reagent Eugene 6 (Roche). Equal numbers of each cell type were seeded for a total of  $3 \times 10^5$  cells per 35 mm dish and grown overnight prior to fusion. The indicated ligands were added to the cells 45 min prior to

fusion, in order to induce nuclear accumulation of the reporter proteins. Cells were incubated at 37°C for 4 hr postfusion in the presence of the indicated nuclear receptor ligands and 10  $\mu$ g/ml cyclohexamide to block protein synthesis. Cells were then fixed and processed for fluorescence microscopy. AR was expressed with an N-terminal FLAG epitope and detected by indirect immunofluorescence using the monoclonal antibody M2 (Sigma) at a dilution of 1:2000. Goat anti-mouse fluorescein conjugated secondary antibody (Pierce) was used at a dilution of 1:100. GR and RAR were expressed as GFP fusions and detected in the FITC channel. The examples shown are representative of 10–25 heterokaryons from two or more independent experiments.

### Microinjection analysis

Cos7 cells were transfected with the indicated vectors and grown overnight in 60 mM dishes;  $1.2 \times 10^5$  cells per 35 mM dish were seeded onto gridded coverslips and grown overnight. Cells were treated with 1  $\mu$ M Dex for 45 min to induce nuclear import of the reporter proteins. Following Dex withdrawal, cells were microinjected with the indicated transport factors and a cascade blue injection marker (1 mg/ml; MW = 70 kDa) using femptotips mounted on a Micromanipulator and Transjector (Eppendorf). Cells were incubated 30 min at 37°C and then fixed and processed for fluorescence microscopy.

### Recombinant proteins

Mouse CRT was subcloned in pQE-30 (Qiagen) for expression as a His-tagged protein in the TG1 *E. coli* strain. Cultures were grown in Luria Broth for 24 hr at 37°C without induction, and His-CRT was purified on TALON beads (Clontech), eluted with imidazole, and dialyzed into 50 mM Hepes (pH 7.4). The expression and purification of His-Crm1 has been described [36]. Recombinant transport factors were flash-frozen in single-use aliquots and stored at –80°C. The nuclear receptor DBDs were expressed in *E. coli* as GST fusions by using pGEX-2T and pGEX-4T vectors (Pharmacia). Cells were grown in Luria Broth, and protein expression was induced when OD<sub>600</sub> reached 0.5, with 1 mM IPTG. The cells were collected by centrifugation, resuspended in lysis buffer (25 mM Tris [pH 7.5] and 150 mM sodium chloride), and sonicated at 5°C until lysis was complete. The lysate was centrifuged at  $15,000 \times g$  for 60 min, and the supernatant was loaded onto a glutathione-Sepharose column. The column was equilibrated and washed extensively with the lysis buffer, and GST-DBDs were eluted with the same buffer supplemented with 10 mM fresh glutathione. The GST-DBD proteins, in some cases, were cleaved with thrombin (Sigma) to generate the free DBDs. The GST and thrombin were removed using a strong cation exchange media SP-Sepharose (Pharmacia). The column was developed with 25 mM Tris buffer and a 150–500 mM sodium chloride gradient, with the GST and thrombin eluting at the beginning of the gradient and the free DBDs eluting at 300–350 mM sodium chloride.

### Binding assays

His-CRT or His-Crm1 (each at 1  $\mu$ g/ml) proteins were incubated for 30 min at 4°C with the indicated GST proteins immobilized on glutathione-Sepharose beads. Binding reactions were carried out in 0.5X transport buffer supplemented with 10 mg/ml bovine serum albumin, 2 mM DTT, and 0.2% Tween-20. Beads were subsequently washed three times, and bound proteins were eluted with SDS-PAGE sample buffer and analyzed by Western blotting. CRT was detected using an anti-CRT rabbit polyclonal antibody (Stressgen), and Crm1 was detected using an anti-Crm1 rabbit polyclonal antibody (kindly provided by Ralph Kehlenbach, Scripps) [37].

### Luciferase assays

Transcriptional reporter activity was measured by using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's directions. NIH3T3 cells were seeded at  $2 \times 10^5$  per well of a 6 well dish and grown overnight prior to transfection. Transfections of the indicated plasmids were performed with the Cytofectene transfection reagent (Bio Rad). The Renilla-luciferase (R-Luc; Promega) and GRE-luciferase (generously provided by Gordon Hager, NIH) reporter vectors were used in all cases. The total input DNA was 1.4  $\mu$ g per well in

each experiment. Transfectants were grown overnight in phenol red-free DMEM containing 10% charcoal stripped fetal bovine serum prior to the indicated Dex treatments. Cells were washed with phosphate-buffered saline and lysed with 300  $\mu$ l passive lysis buffer (Promega). Luciferase activities were measured by using a Berthold LB 953 luminometer.

## Acknowledgements

We thank our University of Virginia colleagues D. Allis, D. Burke, B. Pearson, L. Pemberton, and D. Wotton for reading the manuscript. We also thank G. Hager, J. Hanover, I. Macara, M. Weber, D. Wojchowski, and D. Wotton for their kind gifts of reagents, and M. Skernolis and N. Mou for technical assistance. Financial support from the National Institutes of Health (GM58639-01 to B.M.P.) and the Department of Defense (DAMD 17-00-1-0048 to F.R.) is gratefully acknowledged.

## References

- Evans RM: **The steroid and thyroid hormone receptor superfamily.** *Science* 1988, **240**:889-895.
- Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, et al.: **The nuclear receptor superfamily: the second decade.** *Cell* 1995, **83**:835-839.
- McKenna NJ, Lanz RB, O'Malley BW: **Nuclear receptor coregulators: cellular and molecular biology.** *Endocrinol Rev* 1999, **20**:321-344.
- Hager GL, Lim CS, Elbi C, Baumann CT: **Trafficking of nuclear receptors in living cells.** *J Steroid Biochem Mol Biol* 2000, **74**:249-254.
- Picard D, Yamamoto KR: **Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor.** *EMBO J* 1987, **6**:3333-3340.
- Georget V, Lobaccaro JM, Terouanne B, Mangeat P, Nicolas JC, Sultan C: **Trafficking of the androgen receptor in living cells with fused green fluorescent protein-androgen receptor.** *Mol Cell Endocrinol* 1997, **129**:17-26.
- Nakiely S, Dreyfuss G: **Transport of proteins and RNAs in and out of the nucleus.** *Cell* 1999, **99**:677-690.
- Wente SR: **Gatekeepers of the nucleus.** *Science* 2000, **288**:1374-1377.
- Tyagi RK, Lavrovsky Y, Ahn SC, Song CS, Chatterjee B, Roy AK: **Dynamics of intracellular movement and nucleocytoplasmic recycling of the ligand-activated androgen receptor in living cells.** *Mol Endocrinol* 2000, **14**:1162-1174.
- Guiochon-Mantel A, Lescop P, Christin-Maitre S, Loosfelt H, Perrot-Appinat M, Milgrom E: **Nucleocytoplasmic shuttling of the progesterone receptor.** *EMBO J* 1991, **10**:3851-3859.
- Madan AP, DeFranco DB: **Bidirectional transport of glucocorticoid receptors across the nuclear envelope.** *Proc Natl Acad Sci USA* 1993, **90**:3588-3592.
- Baumann CT, Maruvada P, Hager GL, Yen PM: **Nuclear cytoplasmic shuttling by thyroid hormone receptors. Multiple protein interactions are required for nuclear retention.** *J Biol Chem* 2001, **276**:11237-11245.
- Bunn CF, Neidig JA, Freidinger KE, Stankiewicz TA, Weaver BS, McGrew J, et al.: **Nucleocytoplasmic shuttling of the thyroid hormone receptor  $\alpha$ .** *Mol Endocrinol* 2001, **15**:512-533.
- Komeili A, O'Shea EK: **Nuclear transport and transcription.** *Curr Opin Cell Biol* 2000, **12**:355-360.
- Tyagi RK, Amazit L, Lescop P, Milgrom E, Guiochon-Mantel A: **Mechanisms of progesterone receptor export from nuclei: role of nuclear localization signal, nuclear export signal, and Ran guanosine triphosphate.** *Mol Endocrinol* 1998, **12**:1684-1695.
- Liu J, DeFranco DB: **Protracted nuclear export of glucocorticoid receptor limits its turnover and does not require the exportin 1/CRM1-directed nuclear export pathway.** *Mol Endocrinol* 2000, **14**:40-51.
- Holaska JM, Black BE, Love DC, Hanover JA, Leszyk J, Paschal BM: **Calreticulin is a receptor for nuclear export.** *J Cell Biol* 2001, **152**:127-140.
- Burns K, Duggan B, Atkinson EA, Famulski KS, Nemer M, Bleackley RC, et al.: **Modulation of gene expression by calreticulin binding to the glucocorticoid receptor.** *Nature* 1994, **367**:476-480.
- Dedhar S, Rennie PS, Shago M, Hagesteijn CY, Yang H, Filmus J, et al.: **Inhibition of nuclear hormone receptor activity by calreticulin.** *Nature* 1994, **367**:480-483.
- Wheeler DG, Horsford J, Michalak M, White JH, Hendy GN: **Calreticulin inhibits vitamin D3 signal transduction.** *Nucleic Acids Res* 1995, **23**:3268-3274.
- Omichinski JG, Clore GM, Schaad O, Felsenfeld G, Trainor C, Appella E, et al.: **NMR structure of a specific DNA complex of Zn-containing DNA binding domain of GATA-1.** *Science* 1993, **261**:438-446.
- Katagiri Y, Takeda K, Yu ZX, Ferrans VJ, Ozato K, Guroff G: **Modulation of retinoid signalling through NGF-induced nuclear export of NGFI-B.** *Nat Cell Biol* 2000, **2**:435-440.
- Hsieh JC, Shimizu Y, Minoshima S, Shimizu N, Haussler CA, Jurutka PW, et al.: **Novel nuclear localization signal between the two DNA-binding zinc fingers in the human vitamin D receptor.** *J Cell Biochem* 1998, **70**:94-109.
- Freedman LP, Towers TL: **DNA binding properties of the vitamin D<sub>3</sub> receptor zinc finger region.** *Mol Endocrinol* 1991, **5**:1815-1826.
- McNally JG, Muller WG, Walker D, Wolford R, Hager GL: **The glucocorticoid receptor: rapid exchange with regulatory sites in living cells.** *Science* 2000, **287**:1262-1265.
- DeFranco DB, Qi M, Borror KC, Garabedian MJ, Brautigan DL: **Protein phosphatase types 1 and/or 2A regulate nucleocytoplasmic shuttling of glucocorticoid receptors.** *Mol Endocrinol* 1991, **5**:1215-1228.
- Doucas V, Shi Y, Miyamoto S, West A, Verma I, Evans RM: **Cytoplasmic catalytic subunit of protein kinase A mediates cross-repression by NF- $\kappa$ B and the glucocorticoid receptor.** *Proc Natl Acad Sci USA* 2000, **97**:11893-11898.
- Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, et al.: **Steroid-induced androgen receptor-oestrogen receptor  $\beta$ -SRC complex triggers prostate cancer cell proliferation.** *EMBO J* 2000, **19**:5406-5417.
- Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK: **Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase.** *Nature* 2001, **407**:538-541.
- Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, et al.: **Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity.** *Cell* 2001, **104**:719-730.
- Silver PA, Keegan LP, Ptashne M: **Amino terminus of the yeast GAL4 gene product is sufficient for nuclear localization.** *Proc Natl Acad Sci USA* 1984, **81**:5951-5955.
- McBride KM, McDonald C, Reich NC: **Nuclear export signal located within the DNA-binding domain of the STAT1 transcription factor.** *EMBO J* 2000, **19**:6196-6206.
- Carey KL, Richards SA, Lounsbury KM, Macara IG: **Evidence using a green fluorescent protein-glucocorticoid receptor chimera that the Ran/TC4 GTPase mediates an essential function independent of nuclear protein import.** *J Cell Biol* 1996, **133**:985-996.
- Love DC, Sweitzer TD, Hanover JA: **Reconstitution of HIV-1 Rev nuclear export-independent requirements for nuclear import and export.** *Proc Natl Acad Sci USA* 1998, **95**:10608-10613.
- Walker D, Htun H, Hager GL: **Using inducible vectors to study intracellular trafficking of GFP-tagged steroid/nuclear receptors in living cells.** *Methods* 1999, **19**:386-393.
- Englmeier L, Olivo JC, Mattaj JW: **Receptor-mediated substrate translocation through the nuclear pore complex without nucleotide triphosphate hydrolysis.** *Curr Biol* 1999, **9**:30-41.
- Kehlenbach RH, Dickmanns A, Gerace L: **Nucleocytoplasmic shuttling factors including Ran and CRM1 mediate nuclear export of NFAT in vitro.** *J Cell Biol* 1998, **141**:863-874.
- Black BE, Lévesque L, Holaska JM, Wood TC, Paschal BM: **Identification of an NTF2-related factor that binds Ran-GTP and regulates nuclear protein export.** *Mol Cell Biol* 1999, **19**:8616-8624.
- Black BE, Holaska JM, Lévesque L, Ossareh-Nazari B, Gwizdek C, Dargemont C, et al.: **NXT1 is necessary for the terminal step of Crm1-mediated nuclear export.** *J Cell Biol* 2001, **152**:141-155.



## Ca<sup>2+</sup>-Dependent Nuclear Export Mediated by Calreticulin

James M. Holaska,<sup>1,2†</sup> Ben E. Black,<sup>1,3‡</sup> Fraydoon Rastinejad,<sup>3,4</sup>  
and Bryce M. Paschal<sup>1,3\*</sup>

Center for Cell Signaling<sup>1</sup> and Departments of Microbiology,<sup>2</sup> Biochemistry and Molecular Genetics,<sup>3</sup>  
and Pharmacology,<sup>4</sup> University of Virginia, Charlottesville, Virginia 22908

Received 22 January 2002/Returned for modification 6 March 2002/Accepted 23 May 2002

We have characterized a pathway for nuclear export of the glucocorticoid receptor (GR) in mammalian cells. This pathway involves the Ca<sup>2+</sup>-binding protein calreticulin (CRT), which directly contacts the DNA binding domain (DBD) of GR and facilitates its delivery from the nucleus to the cytoplasm. In the present study, we investigated the role of Ca<sup>2+</sup> in CRT-dependent export of GR. We found that removal of Ca<sup>2+</sup> from CRT inhibits its capacity to stimulate the nuclear export of GR in digitonin-permeabilized cells and that the inhibition is due to the failure of Ca<sup>2+</sup>-free CRT to bind the DBD. These effects are reversible, since DBD binding and nuclear export can be restored by Ca<sup>2+</sup> addition. Depletion of intracellular Ca<sup>2+</sup> inhibits GR export in intact cells under conditions that do not inhibit other nuclear transport pathways, suggesting that there is a Ca<sup>2+</sup> requirement for GR export *in vivo*. We also found that the Ran GTPase is not required for GR export. These data show that the nuclear export pathway used by steroid hormone receptors such as GR is distinct from the Crm1 pathway. We suggest that signaling events that increase Ca<sup>2+</sup> could positively regulate CRT and inhibit GR function through nuclear export.

The nuclear transport machinery integrates a variety of nuclear and cytoplasmic activities by mediating the translocation of housekeeping and regulatory proteins and RNAs. Translocation of these macromolecules generally requires a *cis*-acting transport signal, recognition of the signal by a receptor, and movement of the signal-receptor complex through the nuclear pore complex (NPC) (40, 45). In the case of nuclear export, most proteins rely on a hydrophobic nuclear export signal (NES) and its recognition by the receptor Crm1 (1, 10, 11, 39, 44). NES binding to Crm1 is stabilized by the presence of RanGTP, and the resulting trimeric complex of Crm1, NES, and RanGTP undergoes translocation through the nuclear pore (14, 28). Other proteins are important cofactors for this export pathway, including RanGAP, RanBP1, NXT1, and RanBP3 (1, 4, 22, 24).

A number of proteins that lack a hydrophobic NES are known to undergo nuclear export, and current evidence indicates that three distinct mechanisms can account for nuclear export of these proteins. First, an NES-containing adapter could be used to bridge the interaction between the protein and Crm1 (21). Second, the protein could use a different signal for nuclear export and undergo Crm1-independent export (3). Third, the protein could interact directly with nucleoporins in the NPC and undergo receptor-independent nuclear export (46). An advantage of these mechanisms is that they provide the potential for additional levels of regulation for protein sorting between the nucleus and cytoplasm.

The glucocorticoid receptor (GR) is an example of a protein that undergoes export from the nucleus even though it lacks a hydrophobic NES. Moreover, GR export is insensitive to the Crm1 inhibitor leptomycin B, which seems to rule out the use of NES adapters and Crm1 as the major receptor for this pathway (18, 25). The signal that specifies nuclear export of GR maps to the 67-amino-acid DNA binding domain (DBD), which is both sufficient to mediate the export of green fluorescent protein (GFP) reporter proteins and necessary for export of GR (3). Mutations that disrupt DBD structure, whether in the context of an isolated DBD or in full-length GR, also reduce its export activity. The most severe mutations that reduce DBD-dependent nuclear export are two phenylalanine-to-alanine mutations in the DNA recognition helix (3). The structural conservation of the DBD in the nuclear receptor superfamily suggests that the DBD could be widely used as an export signal. Support for this hypothesis was obtained by showing that the DBDs from steroid, nonsteroid, and orphan nuclear receptors can function as export signals when fused to a GFP reporter protein (3).

Nuclear export mediated by the DBD of GR involves a Ca<sup>2+</sup>-binding protein named calreticulin (CRT), which was first described as a protein in the lumen of the endoplasmic reticulum (ER) (29). The original link between these proteins came with the finding that a peptide sequence, KLGFFKR, which is recognized by CRT, is related to the peptide sequence KVFFKR, which is found in the DNA recognition helix of GR (33). CRT binds to GR and blocks its interaction with DNA in gel shift experiments, and overexpression of CRT inhibits GR-dependent transcription in cells (6). Similar results were obtained when the interactions between the androgen, retinoic acid, and vitamin D receptors and CRT were examined (8). The initial interpretation of these results was that a pool of CRT outside of the lumen of the ER acts as a negative regulator of transcription. The technical difficulty of showing that CRT is out-

\* Corresponding author. Mailing address: Box 800577 Hospital West, Health Sciences System, University of Virginia, Charlottesville, VA 22908. Phone: (434) 243-6521. Fax: (434) 924-1236. E-mail: paschal@virginia.edu.

† Present address: Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

‡ Present address: Ludwig Institute for Cancer Research, University of California, San Diego, CA 92093.

side the ER, however, gave way to the view that CRT is restricted to the ER lumen and that the effects of CRT on gene expression are indirect (23).

Our laboratory purified CRT from HeLa cell cytosol in a search for novel export factors (18). We used a permeabilized cell assay that measures the nuclear export of protein kinase inhibitor (PKI), a protein that contains a hydrophobic NES (19). CRT can bind directly to the NES in PKI, which contains the peptide sequence LALKLAGLDIN. In binding experiments the interaction of CRT with PKI is stabilized in the presence of RanGTP, and in permeabilized cells CRT-dependent export of PKI is enhanced by RanGTP (18). The ability of CRT to act as an export factor for PKI and its prior link with steroid receptor function led us to test whether CRT can function as an export factor for GR. We found that recombinant CRT can stimulate GR export and that the GR export deficiency in *crt*<sup>-/-</sup> cells can be rescued with CRT (18). These observations support the view that CRT functions as an export factor for GR.

In the present study we characterized the CRT-dependent nuclear export of GR, with particular emphasis on how this pathway could be regulated. Because CRT is a Ca<sup>2+</sup> binding protein, we have examined how removing Ca<sup>2+</sup>, either by chelation or by deletion of Ca<sup>2+</sup> binding domains, affects CRT binding to the GR DBD and CRT-dependent nuclear export of GR. We show that Ca<sup>2+</sup> binding to CRT is necessary for direct binding to the DBD and for nuclear export of GR in permeabilized cells. This Ca<sup>2+</sup> requirement involves the C-terminal domain of CRT, which contains multiple low-affinity, high-capacity binding sites for Ca<sup>2+</sup> (2). Removal of the C-terminal domain renders CRT insensitive to Ca<sup>2+</sup> chelation, suggesting that this domain performs a regulatory function that is linked to Ca<sup>2+</sup> binding. While the Ca<sup>2+</sup>-loaded CRT is active for GR export and Ca<sup>2+</sup>-free CRT is inactive for GR export, the opposite is the case for NES export in permeabilized cells. Thus, Ca<sup>2+</sup>-loaded CRT is inactive for Rev export and Ca<sup>2+</sup>-free CRT is active for Rev export. The Ca<sup>2+</sup>-loaded and Ca<sup>2+</sup>-free forms of CRT were shown to have different sensitivities to proteases (7), indicating that CRT adopts different protein conformations. Our data show that these Ca<sup>2+</sup>-dependent conformations of CRT are correlated with the ability to recognize different protein substrates. Ca<sup>2+</sup> depletion in vivo results in the inhibition of GR export, consistent with a Ca<sup>2+</sup> requirement for the translocation of GR from the nucleus to the cytoplasm. Our finding that CRT requires Ca<sup>2+</sup> for binding and nuclear export of GR suggests a potential mechanism for regulating this pathway.

#### MATERIALS AND METHODS

**Plasmids and recombinant proteins.** Standard methods were used for the expression and purification of glutathione *S*-transferase (GST)- and His-tagged proteins, all of which were stored at -80°C as single-use aliquots. The plasmid encoding the GST fusion with CRT was constructed in pGEX4T3, using the open reading frame of mouse CRT lacking the N-terminal 17-amino-acid signal sequence. GST-CRT was expressed in DH5 $\alpha$  bacteria as described previously (18). Full-length and deletion mutants of mouse CRT were cloned into pQE30, and the His-tagged proteins were expressed in TG1 bacteria (3). The wild-type (WT) and NES mutant forms of PKI (L41A, L44A) (44) were expressed in BL21(DE3) bacteria and purified without fusion tags as described previously (19). The plasmid encoding a hydrophobic NES was generated by cloning the DNA sequence that encodes residues 35 to 49 of human PKI into pGEX4T3. Likewise, the plasmid encoding a nuclear receptor DBD was generated by cloning

the DNA sequence that encodes residues 413 to 509 of human GR (3). The GST-NES and GST-DBD proteins immobilized on glutathione beads were either used directly for binding experiments or eluted and used in microtiter plate binding assays. For certain competition experiments, WT and mutant (MUT) forms of the GR DBD (FF to AA) were expressed as GST fusion proteins, cleaved from GST using thrombin, and further purified by ion-exchange chromatography (3). Plasmids encoding His-tagged Ran (WT, Q69L, and T24N; a gift of D. Görlich) and His-tagged Crm1 (a gift of L. Gerace) were used to express proteins in TG1 bacteria, which were purified on Talon resin (Clontech).

**Binding assays.** The three formats used for the solid-phase binding assays were microtiter wells, biosensor cuvettes, and Sepharose beads. The microtiter well assay was performed as described previously (4). Briefly, purified target proteins were immobilized in high-binding 96-well plates (Costar no. 3590) overnight at 4°C in 1 $\times$  transport buffer (30). Unbound protein was removed, and the plates were blocked overnight with bovine serum albumin (30 mg/ml). Binding assays (with 100- $\mu$ l mixtures) were performed in triplicate, using radiolabeled Ran and CRT or Crm1, and the level of binding was measured by scintillation counting as described previously (4). The biosensor assay was performed as described previously (18). Briefly, biotinylated NES peptide was immobilized in streptavidin-coated cuvettes (Fisons) for 15 min at room temperature. The cuvettes were washed with PBS and used for binding assays with the proteins indicated in the legend to Fig. 3. A detailed description of the Fisons biosensor, which measures the change in refractive index that occurs on protein-ligand binding and dissociation, has been published (34). The Sepharose bead binding assays, using either glutathione beads and GST proteins or Talon beads and His-tagged proteins, were carried out by standard methods. Briefly, proteins were immobilized on the beads and blocked overnight with bovine serum albumin (30 mg/ml), and the assays (with 100- $\mu$ l mixtures) were performed using the proteins indicated in the legends. The bound fractions were examined by immunoblotting using antibodies to CRT or Crm1 and enhanced chemiluminescence.

**Ca<sup>2+</sup> removal from CRT.** Ca<sup>2+</sup> was removed from CRT by a published procedure that involves treatment with EGTA (43). Recombinant CRT (0.5 mg/ml in PBS) was incubated for 10 min at 30°C in the presence of 10 mM EGTA, and the sample was then transferred to ice. Ca<sup>2+</sup> was rebound to CRT by supplementing half of the EGTA-treated sample with excess CaCl<sub>2</sub> (final Ca<sup>2+</sup> concentration, 20 mM). The EGTA- and Ca<sup>2+</sup>-treated samples were used at a dilution of at least 1:10 in nuclear export and binding assays, such that the maximum concentration of EGTA in the assays was ~1 mM.

**GR export assays.** Nuclear export of GR in permeabilized cells was performed essentially as described previously (18), except that a stable cell line expressing GR-GFP (a gift of G. Hager) was used instead of transiently transfected cells. The cell line (3676 cells) (27) expresses GR-GFP under the control of a tetracycline-regulated promoter. The cells were grown on glass coverslips for 16 h in the absence of tetracycline to allow GR-GFP expression, and nuclear import of GR-GFP was induced in vivo by dexamethasone (Dex; 1  $\mu$ M) addition to the media. The cells were permeabilized with digitonin (0.005%) for 5 min and used for export assays in vitro with the combinations of transport factors indicated in the legends. At the end of the 20-min export reaction, the samples were washed, fixed, stained with 4',6-diamidino-2-phenylindole (DAPI), and mounted on glass slides. Using a Nikon Microphot SA microscope (60 $\times$  objective, numerical aperture N.A. = 1.40) and a Hamamatsu C-4742-95 charge-coupled device camera, ~50 nuclei from each coverslip were selected using the DAPI channel and DAPI and GFP images were captured. The digital images were acquired using Openlab 2.06 on a Macintosh G3 computer (OS 9.0), and figures were assembled using Adobe Photoshop 5.5 and Freehand 9.0. Mixtures for reactions that measured export and import in the same nuclei contained, in addition to CRT or Crm1, the fluorescent protein allophycocyanin coupled with NLS peptide (APC-NLS) and recombinant import factors (importins and NTF2).

**Hydrophobic NES export assays.** The assay for nuclear export of Rev in permeabilized cells has been described previously (RGG2.2 cells) (18, 26). The fluorescent reporter in this cell line (denoted Rev-GFP) also contains the ligand binding domain of GR, which confers Dex-inducible import in vivo. Digitonin permeabilization, nuclear export, and analysis using fluorescence microscopy were performed as described above for GR-GFP. All export assay mixtures contained Ran, NXT1, and RanBP1. In experiments designed to test the role of Ran in CRT-dependent export, the T24N and Q69L mutant forms of Ran were preloaded with unlabeled GDP and GTP, respectively, and unincorporated nucleotide was removed on a desalting column.

**Ca<sup>2+</sup> depletion in living cells.** Nuclear export of GR was assayed under conditions of Ca<sup>2+</sup> depletion in the 3676 cells by measuring the net redistribution from the nucleus to the cytoplasm. Cells expressing GR-GFP were treated with Dex for 1 h to induce nuclear import and subsequently transferred to phenol red-free media containing ionomycin (Ion; 1  $\mu$ M) or thapsigargin (TG; 1  $\mu$ M) in

the presence of 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-acetoxymethyl ester (BAPTA-AM; 10  $\mu$ M). Cells were also treated with dimethylsulfoxide (DMSO; 0.1%) as a control since the Ion and TG were prepared as 1,000 $\times$  stocks in DMSO. At 0-, 3-, and 6-h time points, the coverslips were fixed in formaldehyde (3.7%) and mounted using Vectashield. The ratio of nuclear to cytoplasmic GR-GFP fluorescence was measured using Openlab software in at least 50 cells per condition. Similar methods were used to assay the effect of  $\text{Ca}^{2+}$  depletion on Rev-GFP export using the RGG2.2 cells.

## RESULTS

**Assembly of CRT complexes in vitro.** In digitonin-permeabilized cells, CRT can stimulate the nuclear export of proteins such as PKI that contain a hydrophobic NES, in a reaction that is dependent on Ran (18). In addition, CRT can stimulate the nuclear export of steroid hormone receptors that lack a hydrophobic NES. The export signal for CRT-dependent export of steroid hormone receptors is contained within the DBD of these proteins (3). The lack of apparent structural similarity between the hydrophobic NES and the DBD led us to characterize how CRT can recognize distinct export signals.

Because Ran is a stoichiometric component of export complexes that contain Crm1 and NES proteins, we first examined whether Ran might also function as a component of export complexes that contain CRT. Previously, we have shown that Ran assembles into a complex containing NES and CRT (18). In the present study, we tested whether Ran can assemble into a complex containing the DBD of GR (amino acids 413 to 509) and CRT. WT PKI, NES MUT PKI, and GST-DBD were immobilized in microtiter wells and incubated with recombinant CRT or Crm1 (5  $\mu$ g each) in the presence of radiolabeled RanGTP ( $2 \times 10^4$  cpm). RanGTP assembled into a complex with CRT or Crm1 in the presence of WT PKI. The reaction is specific since a functional NES in PKI is required for complex assembly. In contrast to these results obtained with the NES-containing protein PKI, RanGTP did not coassemble into a complex with CRT or Crm1 in the presence of the DBD from GR (Fig. 1A). Thus, CRT binding to the DBD is qualitatively different from CRT binding to the hydrophobic NES, since only the latter involves RanGTP as a stoichiometric component.

We carried out binding reactions with the DBD immobilized on glutathione beads and confirmed that CRT binds directly to the DBD and that binding is neither enhanced nor prevented by RanGTP (Fig. 1B). Under the reaction conditions, Crm1 binding to the hydrophobic NES was stimulated by the presence of RanGTP (lane 6). We did not observe Crm1 binding to the DBD in the absence or presence of RanGTP, consistent with our data that Crm1 is not the receptor for proteins that use the DBD as an export signal (18). Our data demonstrate that CRT can assemble into two types of export complexes in vitro: a CRT-NES-RanGTP complex and a CRT-DBD complex.

**Ran-dependent and Ran-independent export by CRT.** The results of our binding assays suggested that CRT-mediated export might occur by both Ran-independent and Ran-dependent mechanisms. That is, the data suggested that nuclear export of hydrophobic NES-containing proteins could be Ran dependent and nuclear export of DBD-containing proteins could be Ran independent. We tested this hypothesis by manipulating the composition and concentration of recombinant export factors in permeabilized cell assays, using GFP fusions of Rev and GR as the export substrates to assay NES- and

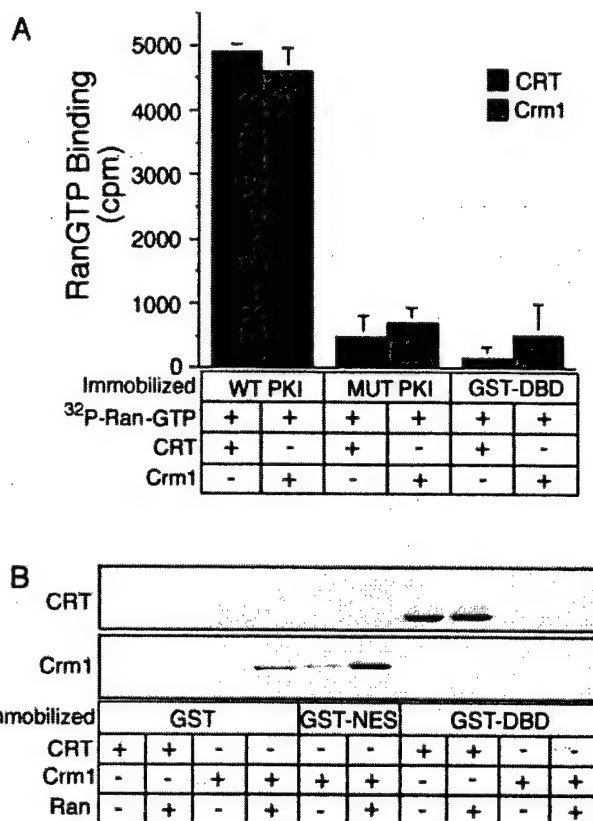


FIG. 1. Formation of export complexes involving CRT and Crm1. (A) The incorporation of RanGTP into complexes containing CRT and Crm1 was assayed using Ran preloaded with [ $\gamma$ - $^{32}\text{P}$ ] GTP. Target proteins were immobilized in microtiter wells (500 ng/well), and CRT or Crm1 (5  $\mu$ g each) was added to each well together with  $2 \times 10^4$  cpm of radiolabeled RanGTP. Following incubation for 1 h at room temperature, the wells were washed four times and the bound fractions were released and assayed by scintillation counting. (B) RanGTP is not a cofactor for CRT binding to the DBD. Target proteins (1  $\mu$ g each) were immobilized on glutathione beads, and CRT or Crm1 (500 ng each) was added to each sample in the absence or presence of Ran (1  $\mu$ g) preloaded with cold GTP. The samples were mixed end over end for 2 h at room temperature, washed three times, eluted, and analyzed by immunoblotting with antibodies to CRT and Crm1. These data show that, like Crm1, NES recognition by CRT involves RanGTP. In contrast, DBD recognition by CRT does not involve RanGTP.

DBD-dependent export, respectively. Both GFP export substrates contain the hormone binding domain of GR, which directs efficient Dex-dependent nuclear import in vivo (31). Following a digitonin permeabilization step, nuclear export of the GFP reporters can be stimulated by the addition of soluble transport factors (26).

We tested for the role of Ran in CRT-dependent NES export by using Rev, which contains a hydrophobic NES (LP-PLERLTL) (10). We used a concentration of CRT that was determined to be subsaturating with respect to Rev-GFP export. In the presence of 0.2  $\mu$ M CRT, WT Ran preloaded with GTP stimulated Rev-GFP export (Fig. 2A). In contrast, the Ran mutant T24N, which mimics the GDP-bound form of Ran, was inactive for CRT-dependent export. The Ran mutant Q69L, which mimics the GTP-bound form of Ran, showed little effect on export mediated by CRT, which is different from



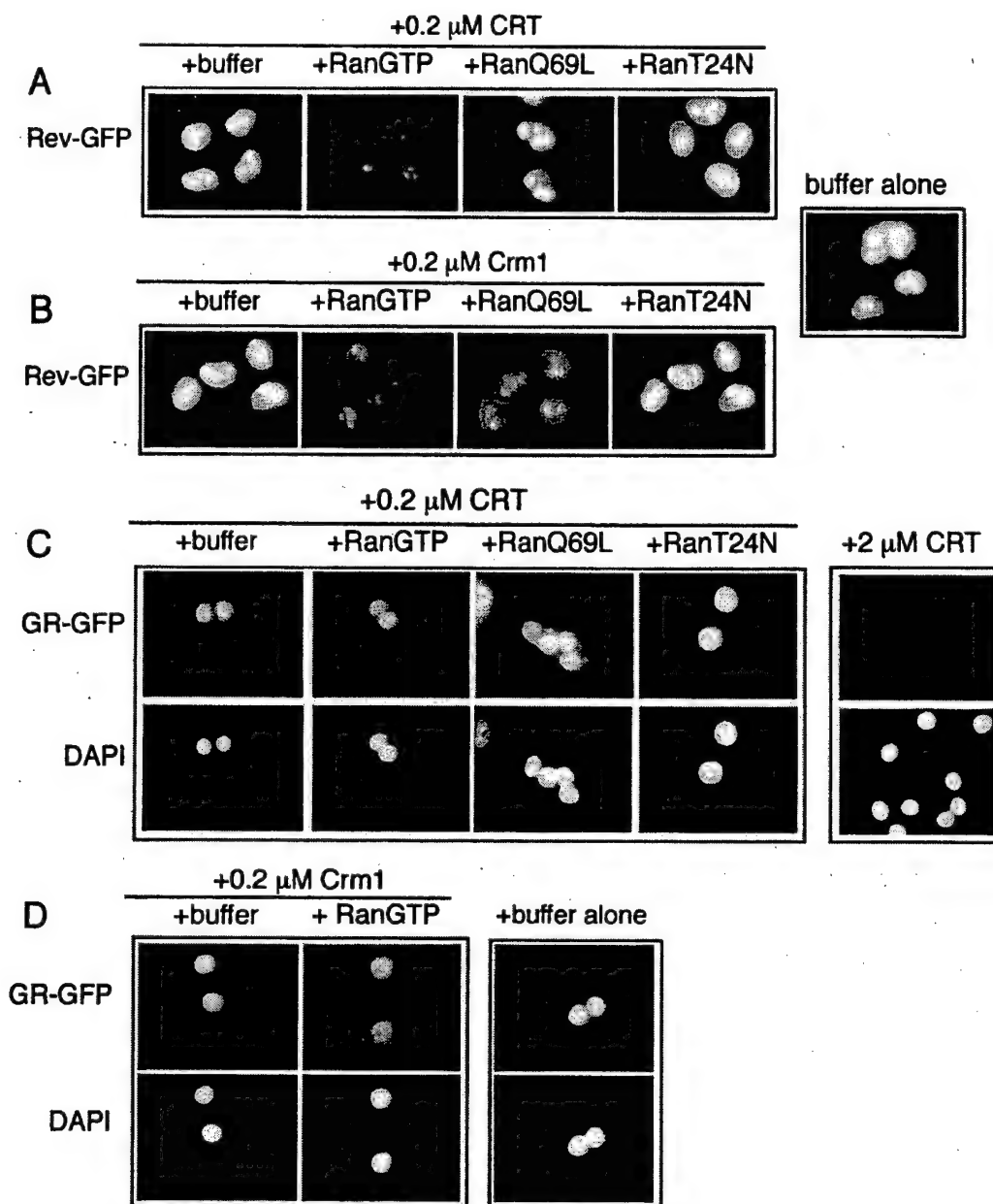


FIG. 2. Ran is a cofactor for NES-dependent export but not for DBD-dependent export. Nuclear export was assayed by supplementing digitonin-permeabilized cell assay mixtures with CRT (0.2 or 2.0  $\mu$ M), Crm1 (0.2  $\mu$ M), and different forms of Ran (1.9  $\mu$ M each). NES-dependent export was assayed using a cell line that expresses a GFP fusion with Rev (RGG2.2) (26), and DBD-dependent export was assayed using a cell line that expresses a GFP fusion with GR (3676) (27). The Rev-GFP fusion also contains the ligand binding domain of GR, which imparts ligand-dependent nuclear import of the reporter protein. Prior to digitonin permeabilization, both cell lines were treated with Dex (1  $\mu$ M) to induce nuclear import of the GFP reporters. (A and B) RanGTP stimulates Rev-GFP export in the presence of CRT or Crm1. (C and D) In contrast, neither WT nor MUT forms of Ran stimulate GR-GFP export in the presence of CRT or Crm1.

previous results obtained using another RanGTP mutant (G19V) and a fluorescent conjugate of PKI as the export substrate (18). The reason for this discrepancy is unclear, but it could relate to subtle differences in the structure and activity of these mutant proteins or to the fact that different NES proteins were used in the two assays, or both. Crm1 in these assays (Fig. 2B) supports robust export of Rev-GFP in the presence of WT Ran preloaded with GTP. In the presence of the Ran mutant Q69L, a moderate level of export was observed, while the Ran

mutant T24N failed to support Crm1-dependent export. Thus, Ran stimulates both CRT- and Crm1-dependent export in this system, and maximal export activity of either protein is observed only in the presence of WT Ran.

GR-GFP export was assayed at the same concentration of CRT (0.2  $\mu$ M) that, in the presence of RanGTP, promotes efficient export of Rev GFP. We determined that the addition of subsaturating CRT together with Ran does not support nuclear export of GR. We also found that the addition of Crm1

and RanGTP at concentrations (0.2 and 1.9  $\mu$ M, respectively) that promote efficient Rev export fails to support GR export. These transport data correlate with our *in vitro* binding data, since CRT-dependent binding and export of an NES-containing protein is dependent on RanGTP. In contrast, CRT-dependent binding and export of the DBD appears to be independent of Ran. In the context of permeabilized cell assays, CRT can substitute for Crm1 in the NES pathway; however, Crm1 cannot substitute for CRT in the DBD pathway.

**Overlapping export substrate binding sites on CRT.** We considered two models to account for the ability of CRT to bind and mediate the nuclear export of two substrates with apparently unrelated export signals. In the first model, CRT could contain two distinct substrate binding sites, with only one of the two sites regulated by RanGTP. In the second model, CRT could contain a single substrate-binding site that accommodates both types of substrate and could use RanGTP to regulate the binding of only one of the two types of substrate. We reasoned that if CRT uses a single substrate binding site (or a single type of binding site), then CRT binding to the DBD should be inhibited in the presence of excess NES. To address this issue, we assayed CRT binding to an immobilized DBD in the absence and presence of WT and NES mutant forms of PKI. CRT binding to the DBD was reduced by the presence of WT PKI but not by the NES mutant form of PKI (Fig. 3A). Our data are consistent with a single type of substrate binding site on CRT, although we cannot rule out the possibility that CRT contains a second substrate binding site that is inhibited by an allosteric mechanism.

We also tested whether CRT contains a single type of substrate binding site by assaying nuclear export in permeabilized cells. Nuclear export of GR-GFP was tested in the absence and presence of WT and NES mutant forms of PKI. Inclusion of excess WT PKI (12  $\mu$ M) in the reaction mixture inhibited the nuclear export of GR, whereas inclusion of the NES mutant form of PKI at the same concentration had no effect (Fig. 3B). GR export mediated by CRT was blocked by excess GR DBD (4.5  $\mu$ M), consistent with previous data showing that the DBD functions as the signal for nuclear export of GR (3, 18).

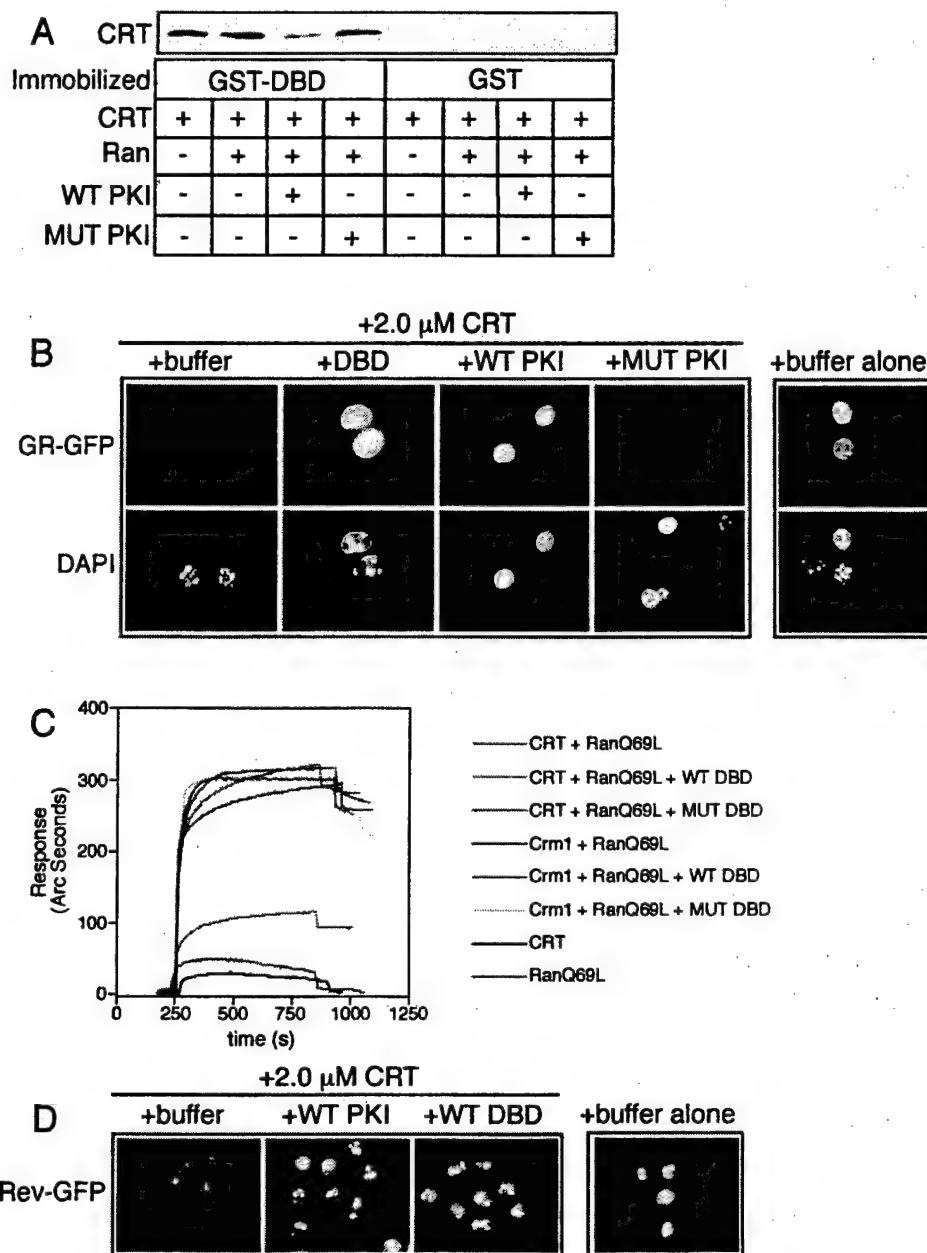
The assays described above (Fig. 3A and B) were designed to measure the effect of excess NES on CRT binding to, and export of, the DBD. We performed analogous protein binding and nuclear export experiments that, instead, measured the effect of excess DBD on the interaction between CRT and the NES (Fig. 3C). In the biosensor assay, CRT binding to the NES (green tracing) or Crm1 binding to the NES (purple tracing) were both observed, but only in the presence of RanGTP. CRT binding to the NES was reduced markedly by the presence of a WT DBD (light blue tracing), but this interaction was unaffected by the presence of a MUT DBD (dark blue tracing). The ability of the DBD to compete with NES for binding to CRT provides evidence that a single type of binding site is used for both substrates.

The effect of excess DBD on CRT-dependent export of NES substrate was tested in permeabilized cells by using Rev-GFP as a reporter. CRT-dependent export of Rev-GFP was inhibited by excess DBD and, as expected, by excess WT PKI (Fig. 3D). Addition of excess WT PKI resulted in inhibition of Crm1-dependent export of Rev-GFP, while addition of excess DBD had no effect on Rev-GFP export (data not shown). In

summary, our results demonstrate that (i) CRT can bind to and mediate the nuclear export of a protein that contains either a hydrophobic NES or a steroid hormone receptor DBD, (ii) CRT-dependent binding and export of a protein containing the hydrophobic NES is dependent on Ran, (iii) CRT-dependent binding and export of a protein containing an appropriate DBD is not dependent on Ran, and (iv) CRT uses a similar substrate binding site for proteins that contain either a hydrophobic NES or an appropriate DBD.

**Ca<sup>2+</sup> is critical for CRT export activity.** CRT was originally discovered as a Ca<sup>2+</sup> binding protein (29), and many of its functions in the ER have been proposed to be linked to its ability to bind Ca<sup>2+</sup> (20). To determine if Ca<sup>2+</sup> influences the nuclear export activity of CRT, we used an established method to release Ca<sup>2+</sup> from recombinant CRT (43) and tested the protein in binding and transport assays. EGTA-induced Ca<sup>2+</sup> release from CRT resulted in the loss of CRT binding to the DBD. Binding to the DBD was, however, restored by the addition of excess Ca<sup>2+</sup> (Fig. 4A). Ca<sup>2+</sup> removal from CRT caused a corresponding reduction in its capacity to promote nuclear export of GR in permeabilized cells, and the export activity was restored by the addition of excess Ca<sup>2+</sup> (Fig. 4B). Unexpectedly, the capacity of CRT to mediate nuclear export of the NES substrate Rev-GFP was unaffected by EGTA, and the addition of excess Ca<sup>2+</sup> to this assay actually inhibited Rev-GFP export (Fig. 4C). The effect of Ca<sup>2+</sup> on Rev-GFP export appears to be linked to CRT since these treatments did not affect Crm1-dependent export of Rev-GFP (Fig. 4D). These data suggest that the Ca<sup>2+</sup>-bound state of CRT determines whether it binds to DBD- or NES-containing proteins and that these interactions are mutually exclusive under the conditions used in our assays.

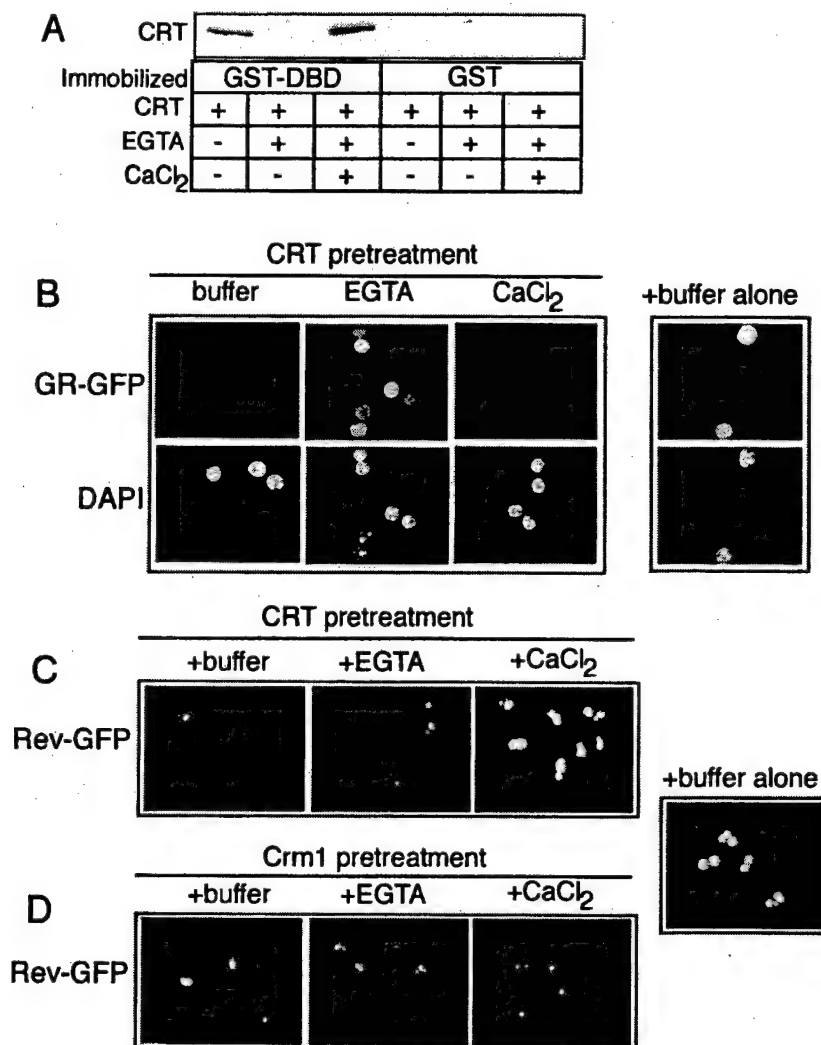
We considered it formally possible that addition of EGTA to digitonin-permeabilized cells could block GR export by inhibiting NPC function. Ca<sup>2+</sup> depletion in cells has been reported to inhibit the nuclear import of an NLS reporter protein (15), and there is structural evidence that depletion of Ca<sup>2+</sup> from the lumen of the nuclear envelope can alter NPC structure (41). It was also possible that Ca<sup>2+</sup>-free CRT might accumulate at the NPC and block transit through the nuclear pore. We addressed this issue in a permeabilized cell assay that reconstitutes both nuclear import and export (Fig. 5A), reasoning that nuclear import would provide a relevant readout of NPC activity that is independent of nuclear export. Import in these assays was reconstituted by the addition of  $\alpha$ - and  $\beta$ -importin, Ran, and NTF2 and was monitored by the nuclear accumulation of allophycocyanin conjugated with the simian virus 40 large T-antigen NLS (APC-NLS). EGTA effectively blocked GR export without affecting APC-NLS import (Fig. 5B). Moreover, excess Ca<sup>2+</sup> blocked CRT-dependent export of Rev-GFP without affecting APC-NLS import. This effect was specific to CRT since Crm1-dependent export of Rev-GFP was unaffected by excess Ca<sup>2+</sup> (Fig. 5C). Our data are consistent with Ca<sup>2+</sup>-mediated regulation of the activity of CRT and not with the inactivation of NPC function. We note that our standard assay buffer contains 1 mM EGTA. Because this condition is permissive for CRT-dependent export, removal of Ca<sup>2+</sup> from CRT may require the higher concentration of EGTA (10 mM) or pretreatment of purified CRT with EGTA at 30°C, or both.



**FIG. 3.** The DBD and hydrophobic NES use a common or overlapping binding site on CRT. (A) Binding assay with GST-DBD or GST (2  $\mu$ g each) immobilized on glutathione beads and CRT (500 ng), RanGTP (2  $\mu$ g), and WT or MUT PKI (2  $\mu$ g) added in solution. The bound fractions were analyzed by immunoblotting for CRT. Including WT PKI in the reaction reduced the level of CRT bound to the DBD, indicating that these proteins bind to similar sites on CRT. This competition was not observed when RanGTP was omitted from the assay (data not shown), consistent with Ran acting as a cofactor for NES binding but not for DBD binding. (B) Nuclear export of GR-GFP was assayed in permeabilized cells using CRT (2.0  $\mu$ M) in the presence of buffer, excess DBD (4.5  $\mu$ M), or PKI (12  $\mu$ M WT or MUT). (C) Competitive binding interactions between NES, DBD, and CRT measured in a biosensor assay. NES peptide was immobilized on the cuvette surface through a biotin-neutravidin linkage and was used to measure the Ran-dependent binding of CRT in the absence and presence of DBD in the solution. The proteins used in the assay were CRT (1.1  $\mu$ M), Crm1 (1.1  $\mu$ M), RanQ69L (1.9  $\mu$ M), and WT and MUT DBD from GR (9.1  $\mu$ M each). CRT binds efficiently to NES peptide in the presence of Ran (green tracing), and this can be competed with the WT DBD (light blue tracing) but not with the transport-defective MUT DBD that contains the FF-to-AA mutations (dark blue tracing). Crm1 binding to the NES is unaffected by the presence of excess DBD (fuchsia tracing). (D) The DBD competes with NES in the CRT-dependent export pathway. The cell line expressing Rev-GFP was used to assay export in the presence of CRT (1.1  $\mu$ M), WT PKI (12  $\mu$ M), and WT DBD (9.1  $\mu$ M) as indicated.

**The P-domain and C-terminal domain of CRT Impart  $\text{Ca}^{2+}$  regulation.** As diagrammed in Fig. 6A, the domain structure of CRT includes an acidic C-terminal domain that contains multiple low-affinity, high-capacity  $\text{Ca}^{2+}$  binding sites ( $K_D \approx 2$

mM;  $>25$  mol/mol) and a proline-rich P-domain that contains high-affinity, low-capacity  $\text{Ca}^{2+}$  binding sites ( $K_D \approx 1$   $\mu$ M) (2). In light of our data showing that  $\text{Ca}^{2+}$  can regulate CRT-dependent export of GR, we examined whether the C-terminal



**FIG. 4.** Ca<sup>2+</sup> binding to CRT is necessary for nuclear export of GR. (A) Binding assay performed with GST-DBD or GST (2.5  $\mu$ g each) immobilized on glutathione beads and CRT (500 ng). The amount of CRT bound in each reaction fraction was examined by immunoblotting for CRT. The CRT used in the binding assay was untreated, pretreated with 10 mM EGTA, or pretreated with 10 mM EGTA and 20 mM CaCl<sub>2</sub>. Ca<sup>2+</sup> removal from CRT inhibits binding to the DBD, and this can be reversed by addition of Ca<sup>2+</sup>. (B and C) Ca<sup>2+</sup> is required for CRT-dependent GR export; however, Ca<sup>2+</sup> inhibits CRT-dependent NES export. (D) The presence of excess EGTA and Ca<sup>2+</sup> in the permeabilized-cell assay mixture does not affect the export mediated by Crm1. CRT and Crm1 were used at a final concentration of 1.1  $\mu$ M each in the export assays. The pretreatment of CRT and Crm1 with EGTA is described in Materials and Methods.

domain is required for CRT activity in our assays. CRT mutants lacking a portion of the C-terminal domain (retaining residues 1 to 350) or the entire C-terminal domain (retaining residues 1 to 273) were still functional for nuclear export of Rev-GFP, and the activities of these mutants was blocked by excess Ca<sup>2+</sup> (Fig. 6B to D). From these data we infer that the Ca<sup>2+</sup>-dependent inhibition of hydrophobic NES export by CRT can be ascribed to the high-affinity low-capacity Ca<sup>2+</sup> binding site in the P-domain.

The deletion mutants were also examined for the Ca<sup>2+</sup> dependence of DBD binding and GR export. We found that the deletion mutants still bound to the DBD in the presence of Ca<sup>2+</sup> and that the mutant (residues 1 to 273) lacking the entire C-terminal domain was able to bind to the DBD in the presence of EGTA, albeit to a lesser extent than was the full-length CRT (Fig. 6E). This suggests that in the absence of Ca<sup>2+</sup>, the

C-terminal domain normally exerts a negative regulation on the substrate binding activity of CRT. Removal of the entire C-terminal domain (residues 1 to 273) also resulted in loss of the EGTA-dependent inhibition of GR export that is observed with both the full-length and partial C-terminal domain deletion mutant (Fig. 6F to H). These data indicate that the low-affinity, high-capacity Ca<sup>2+</sup> binding sites in the C-terminal domain of CRT are not required for nuclear export of hydrophobic NES or DBD-containing proteins but that they do contribute a regulatory function. The Ca<sup>2+</sup> binding sites in the C-terminal domain are important for the EGTA-sensitive GR export, and the Ca<sup>2+</sup> binding site in the P-domain appears to be sufficient to confer Ca<sup>2+</sup>-induced inhibition of NES export.

**GR-GFP Export is Sensitive to Ca<sup>2+</sup> Depletion.** Our results showing that GR undergoes CRT- and Ca<sup>2+</sup>-sensitive export in permeabilized cells prompted us to examine whether this

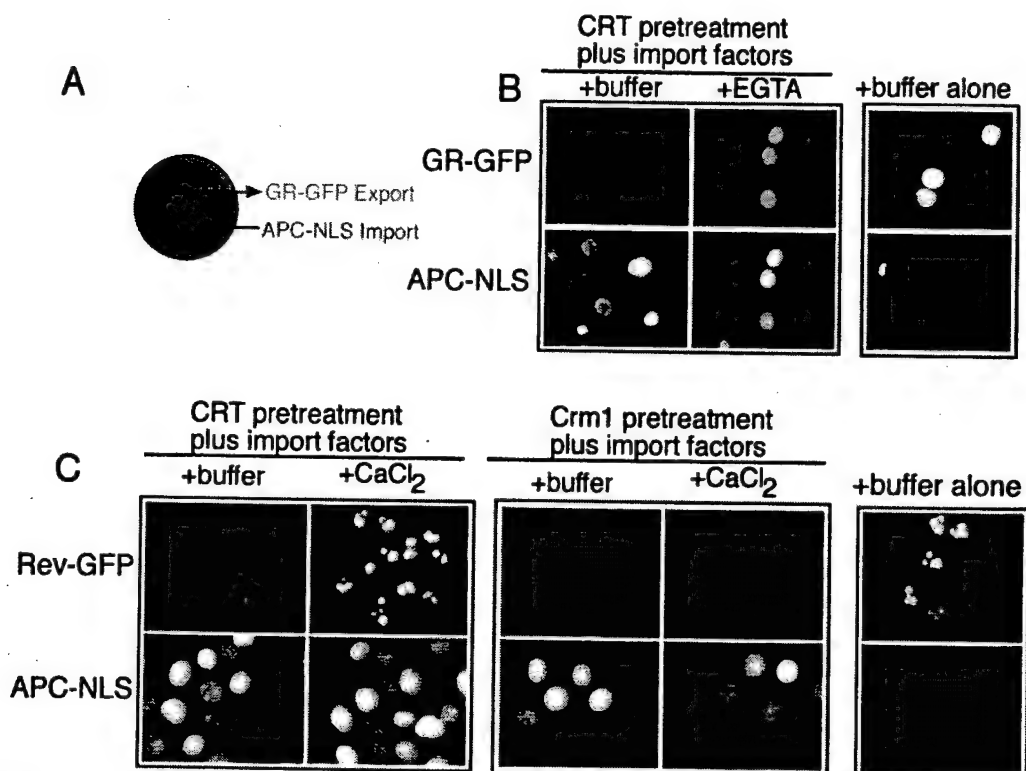


FIG. 5.  $\text{Ca}^{2+}$  chelation with EGTA inhibits CRT-dependent export without affecting nuclear import in permeabilized cells. (A) Diagram illustrating the assay for nuclear import (APC-NLS) and export (GR-GFP) in the same cells. (B) EGTA blocks CRT-dependent export of GR-GFP without affecting the nuclear import of APC-NLS in the presence of  $\beta$ -importin, Rch1, Ran, NTF2, NXT1, and RanBP1 (50  $\mu\text{g}/\text{ml}$  each). (C) Excess  $\text{Ca}^{2+}$  blocks CRT-dependent export of Rev-GFP without affecting the nuclear import of APC-NLS.

pathway is operational in living cells. The 3676 cell line (27), which stably expresses GR-GFP, was treated with Dex to induce import and then transferred to Dex-free medium that contained reagents known to deplete luminal and cytosolic  $\text{Ca}^{2+}$ . The effect of  $\text{Ca}^{2+}$  depletion on nuclear export was examined by fluorescence microscopy after 6 h and quantitated by measuring the nuclear/cytoplasmic fluorescence ratio of GR-GFP. The conditions included treatment with the  $\text{Ca}^{2+}$  ionophore Ion (1  $\mu\text{M}$ ) or the ER  $\text{Ca}^{2+}$  pump inhibitor TG (1  $\mu\text{M}$ ). The membrane-permeable form of BAPTA-AM (10  $\mu\text{M}$ ) was included with Ion and TG to chelate  $\text{Ca}^{2+}$  that was released. We found that Ion or TG administered in the presence of BAPTA-AM were both effective at blocking the nuclear export of GR-GFP (Fig. 7A). During the export phase of the experiment, the nuclear/cytoplasmic fluorescence in the control cells showed a significant reduction from  $11.9 \pm 2.1$  to  $1.5 \pm 1.5$  (Fig. 7B). In contrast, the nuclear/cytoplasmic fluorescence in the Ion- and TG-treated cells showed only a slight reduction, to  $9.9 \pm 1.2$  and  $10.5 \pm 0.9$ , respectively.

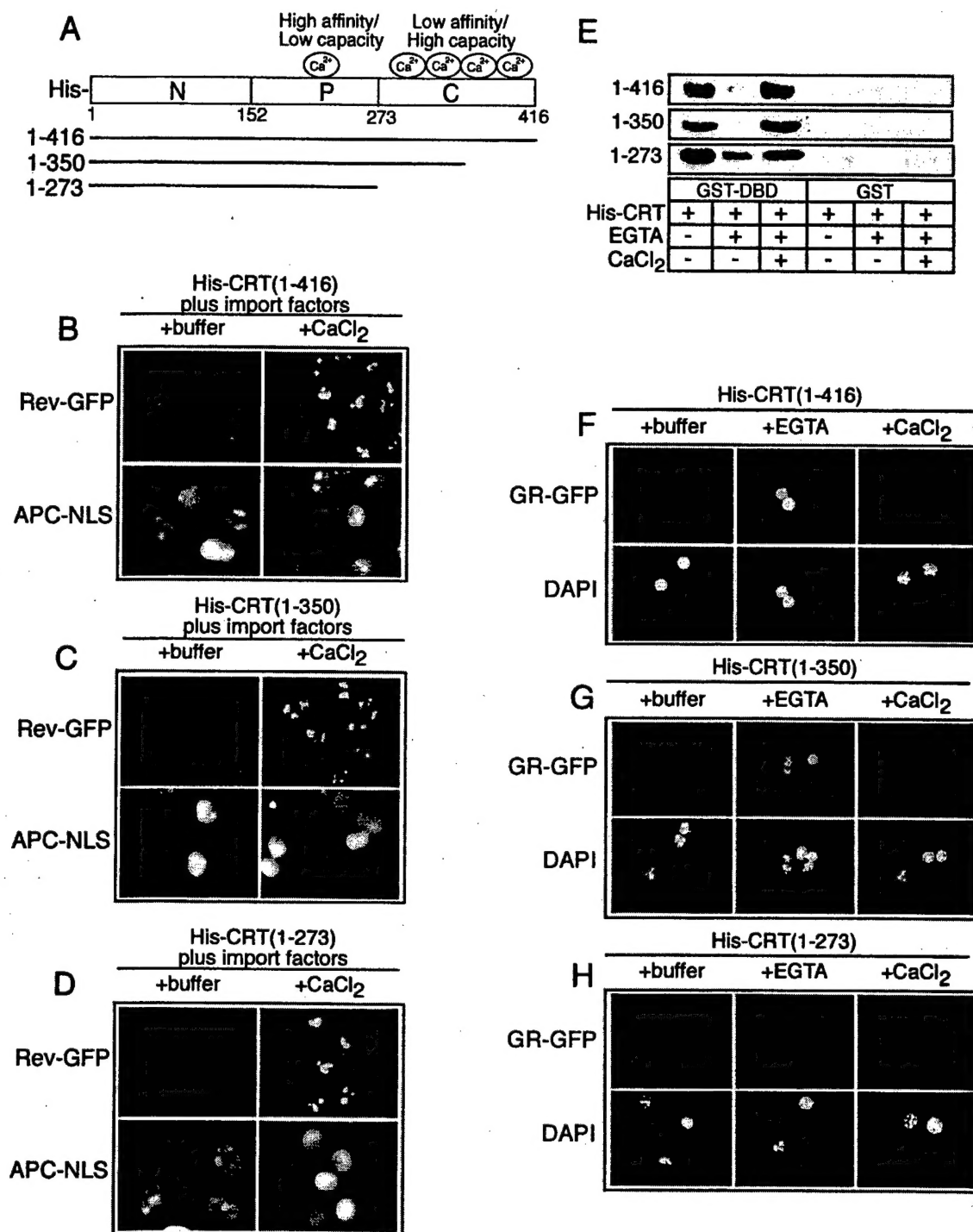
It is unlikely that Ion, TG, and BAPTA-AM cause a global disruption of NPC structure because NES-dependent export of Rev-GFP continues under  $\text{Ca}^{2+}$  depletion conditions in vivo (Fig. 7C) as well as in vitro (26). The transport of other cargos is unaffected by  $\text{Ca}^{2+}$  depletion in vivo, including Crm1-dependent nuclear export of mitogen-activated protein kinase-activated protein kinase 2 (42) and importin/karyopherin-dependent nuclear import of Rev-GFP (B. Black, unpublished observations). Our finding that nuclear export of GR in per-

meabilized cells is sensitive to the  $\text{Ca}^{2+}$ -bound state of the CRT used in the assays suggests that depletion of  $\text{Ca}^{2+}$  in vivo blocks the CRT-dependent export of GR in the cell.

## DISCUSSION

Our study has revealed that  $\text{Ca}^{2+}$  and RanGTP can regulate the nuclear export activity of CRT.  $\text{Ca}^{2+}$  binding to CRT is necessary for its interaction with the DBD of GR in solid-phase binding assays and for nuclear export of GR in digitonin-permeabilized cell assays. These interactions appear to be independent of Ran, since Ran is not required for CRT binding to the DBD. Ran does not stimulate GR export when CRT is rate-limiting in the assay, and GTPase-deficient mutants of Ran do not block GR export. Under conditions where the low-affinity  $\text{Ca}^{2+}$  binding sites of CRT are saturated with  $\text{Ca}^{2+}$ , CRT does not support the nuclear export of a hydrophobic NES substrate. The export capacity for a hydrophobic NES substrate can be restored by EGTA-mediated removal of  $\text{Ca}^{2+}$  from CRT. Under these conditions of low  $\text{Ca}^{2+}$ , RanGTP acts as a stimulatory factor of CRT in a mechanism that involves the assembly of CRT, NES, and RanGTP into a complex (18). Collectively, our data suggest that  $\text{Ca}^{2+}$  binding determines whether CRT interacts with DBD containing cargo or with NES-containing cargo, and only the latter involves Ran.

The characterization of CRT over the past several years has revealed that it can associate with structurally distinct substrates (20). Studies of the chaperone-like functions of CRT



**FIG. 6.** The low-affinity  $\text{Ca}^{2+}$  binding sites in the C-terminal domain of CRT are not essential for nuclear export activity. (A) Diagram of the CRT structure and sites of  $\text{Ca}^{2+}$  binding. (B to D) Export assays performed with CRT proteins containing deletions in the low-affinity  $\text{Ca}^{2+}$  binding C-terminal domain. CRT lacking the entire C-terminal domain retains its  $\text{Ca}^{2+}$ -dependent inhibition of NES export, indicating that this is probably due to the high-affinity, low-capacity  $\text{Ca}^{2+}$  binding site. (E) EGTA-sensitive binding of CRT to the DBD is partially lost on removal of the C-terminal domain (residues 1 to 273). (F to H) Removal of the entire C-terminal domain (residues 1 to 273) from CRT abrogates the EGTA-sensitive export of GR. Although the C-terminal domain of CRT is not required for export, it is necessary for  $\text{Ca}^{2+}$  regulation of GR export.

proposed to occur in the ER lumen have addressed the *in vitro* interaction of CRT with oligosaccharides, both free in solution and as a structural component of glycoproteins. CRT is capable of binding directly to high-mannose-containing oligosac-

charides, and CRT can partially suppress the aggregation of certain glycosylated proteins exposed to elevated temperatures (35). It can also suppress the thermal aggregation of nonglycosylated proteins (35). These data have been taken as evi-



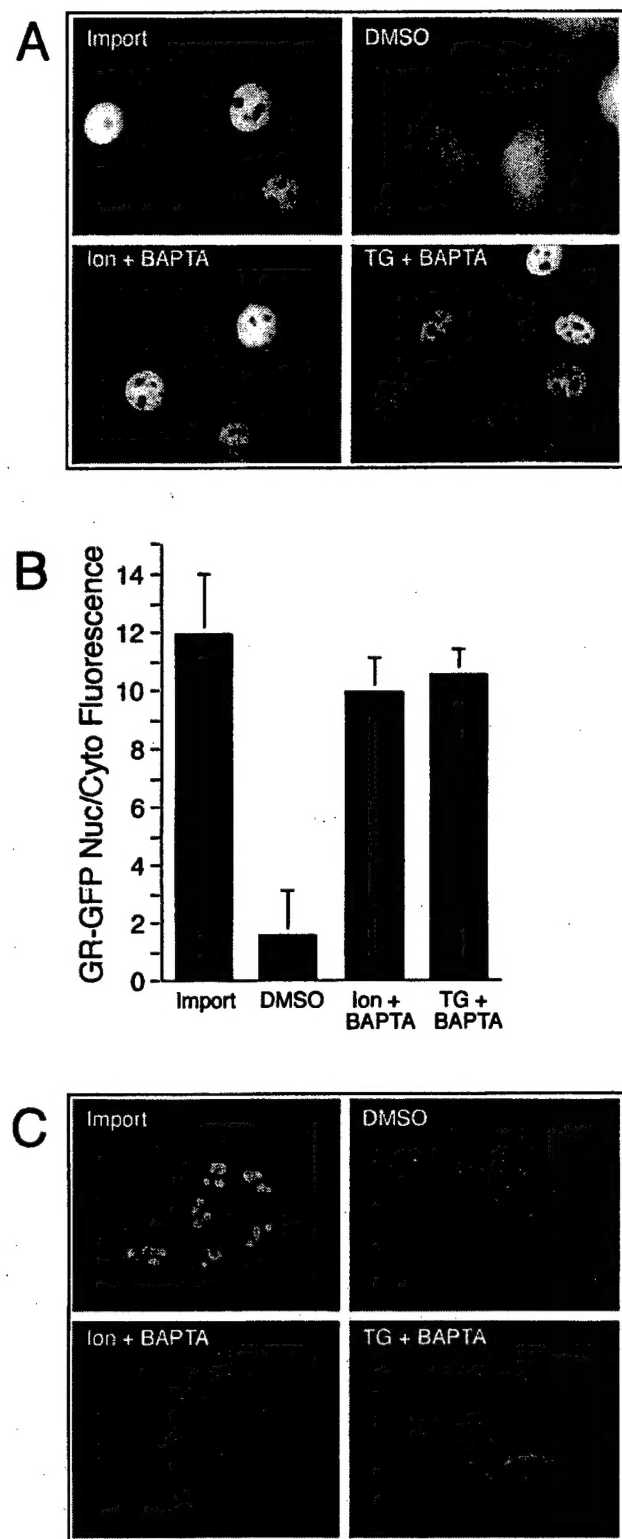


FIG. 7.  $\text{Ca}^{2+}$  depletion in vivo inhibits the nuclear export of GR. (A) Representative fields of cells expressing GR-GFP after induction of nuclear import with Dex (Import). Following Dex removal, the cells were treated for 5 h with vehicle (DMSO), Ion and BAPTA-AM, or TG and BAPTA-AM, and the GR-GFP distribution was recorded in living cells. (B) Measurements of the nuclear/cytoplasmic (Nuc/Cyto) ratios of GR-GFP fluorescence in cells incubated under conditions that deplete  $\text{Ca}^{2+}$ . Depletion of luminal stores of  $\text{Ca}^{2+}$  with Ion and

dence that in the ER, CRT may use both its lectin and peptide binding properties to stabilize protein-folding intermediates. The view that CRT participates in the folding of glycoproteins in the ER is logical, given the sequence relatedness (34% identity; expectation value,  $<10^{-50}$ ) and functional similarities to the ER chaperone calnexin. Calnexin is a key component of a quality control pathway that monitors the folding state of glycoproteins in the ER. The current view is that calnexin and CRT interact transiently with protein-folding intermediates, effectively retaining them in the ER until a native conformation is attained (17).

Studies of the functions of CRT that occur outside the ER have yielded an even more diverse collection of substrates (20). These include the cytoplasmic domain of  $\alpha$ -integrins (33), steroid hormone receptors (6), NES-containing proteins (18), and a stem-loop structure from rubella virus RNA (38). Some of these substrates contain a binding site for CRT that can be recognized at the sequence level, while other substrates seem structurally distinct. The site within  $\alpha$ -integrin recognized by CRT is the sequence KXGFFKR, which is similar to the sequence KVFFKR within the DNA recognition helix of GR and other nuclear receptors. The NESs in PKI (LALKLAGLDIN) and Rev (LPPLRLTL) are similar to each other, but they show little resemblance to the CRT binding sites in  $\alpha$ -integrin and the DNA recognition helix. One feature that is shared by most of these signals is the presence of hydrophobic amino acids that, when mutated, abrogates binding to CRT. In the case of the hydrophobic NES, the critical leucines are probably part of an amphipathic helix and the side chains are predicted to physically contact the receptors Crm1 and CRT. This is not the case for the two phenylalanines within the DNA recognition helix that are necessary for binding to CRT (3). The crystal structures of different nuclear receptors reveal that the phenylalanine side chains pack against the core of the DBD, at least when bound to DNA (32). Thus, the FF-to-AA mutations in the DNA recognition helix of GR that inhibit nuclear export probably affect the folding of the DBD, resulting in a structure that is no longer recognized by CRT (3).

How does CRT recognize its substrates? Our present study and previous work from other laboratories suggest that the N-terminal domain mediates substrate recognition and that regulation of binding is imparted by the  $\text{Ca}^{2+}$ -dependent conformational changes in the P-domain and C-terminal domain of CRT. Multiple low-affinity  $\text{Ca}^{2+}$  binding sites are contained within the C-terminal domain of CRT, and at least one high-affinity  $\text{Ca}^{2+}$  binding site is contained in the middle domain (P-domain) (2).  $\text{Ca}^{2+}$  is a cofactor for CRT binding to oligosaccharides, glycoproteins, and certain nonglycosylated proteins in vitro (43). Moreover, in our assays,  $\text{Ca}^{2+}$  is necessary for the physical interaction between CRT and the DBD as

TG and chelation with BAPTA-AM results in significant reduction of GR export to the cytoplasm. (C) Depletion of luminal  $\text{Ca}^{2+}$  stores does not inhibit NES export. The RGG 2.2 cell line expressing Rev-GFP was treated with Dex to induce importing of the reporter, which concentrates in the nucleoli. The cells were then maintained for 6 h under conditions that deplete  $\text{Ca}^{2+}$ . During the last 1 h, Dex was removed to allow Rev-GFP export to the cytoplasm. Rev-GFP export was observed whether or not  $\text{Ca}^{2+}$  was depleted.

measured by direct binding and as assayed by nuclear export of GR in permeabilized cells.

$\text{Ca}^{2+}$  binding induces structural changes in CRT that dramatically alter its fragmentation pattern that results when it is treated with several different proteases (7). Of particular relevance to our study is the N-terminal 27 kDa of CRT, which is protected from tryptic digestion in the presence of  $\text{Ca}^{2+}$  but is highly susceptible in the absence of  $\text{Ca}^{2+}$  (7). This protease-resistant core of CRT retains at least one high-affinity  $\text{Ca}^{2+}$  binding site from the P-domain, while all of the low-affinity  $\text{Ca}^{2+}$  binding sites in the C-terminal domain are removed by digestion. We found that a CRT deletion mutant (residues 1 to 273) that contains the protease-resistant core is still functional for nuclear export of GR in permeabilized cells, indicating that the low-affinity  $\text{Ca}^{2+}$  binding sites are dispensable for our assays that score a positive interaction between CRT and GR. It is interesting that this CRT deletion mutant (residues 1 to 273) shows some  $\text{Ca}^{2+}$ -independent binding to the DBD, although the high-affinity  $\text{Ca}^{2+}$  binding site is still present in this mutant. The fact that the C-terminal  $\text{Ca}^{2+}$  binding sites are necessary for EGTA-induced inhibition of CRT binding to the DBD strongly suggests that the C-terminal domain functions in negative regulation of substrate binding. We have also observed that the N-terminal domain of CRT (residues 1 to 150) expressed as a GST fusion protein is sufficient to mediate nuclear export of GR in permeabilized cells (unpublished observations). This is consistent with previous data showing that the N-terminal domain of CRT can block GR binding to the GRE in vitro (6). All available information indicates, therefore, that the N-terminal domain of CRT is the substrate binding domain that physically contacts the DBD of GR. We infer that the N-terminal domain also binds hydrophobic NES substrates, since the DBD and NES display competitive interactions with CRT in our binding assays.

Emerging structural information provides a context for interpreting how  $\text{Ca}^{2+}$  might regulate CRT activity. Although the atomic structure of CRT has not yet been solved, the structure of the luminal domain of calnexin, which is 35% identical to CRT, has been solved at 2.9 Å resolution (37). The structure of calnexin reveals that it contains a compact globular domain (residues 61 to 262 and 415 to 458) and a large hairpin that forms a highly extended arm (residues 277 to 410). Tandem repeats in the P-domain comprise the arm, which projects 140 Å away from globular domain. The hairpin structure brings the N- and C-terminal domains together to form the globular domain (37). Thus, the close proximity of these domains may help explain how the occupancy of  $\text{Ca}^{2+}$  binding sites in the C-terminal domain can influence substrate recognition by the N-terminal domain in both calnexin and CRT. The nuclear magnetic resonance spectroscopy structure of the P-domain of CRT (residues 189 to 288) was recently solved, and, like calnexin, this domain forms a highly extended arm (9). The crystal structure of calnexin and the nuclear magnetic resonance spectroscopy structure of the P-domain of CRT were both solved in the presence of  $\text{Ca}^{2+}$ , and so the basis of  $\text{Ca}^{2+}$ -dependent changes in structure awaits further studies. It has been suggested that the P-domain or arm is a  $\text{Ca}^{2+}$ -sensitive protein interaction site for CRT (9).

Does  $\text{Ca}^{2+}$  regulate the engagement of CRT with nuclear export substrates? Both cytoplasmic and nuclear  $\text{Ca}^{2+}$  levels

can respond to signaling events, and in some cases the modulation of nuclear  $\text{Ca}^{2+}$  occurs independently of cytoplasmic  $\text{Ca}^{2+}$  (36). Depending on the system and method of measurement,  $\text{Ca}^{2+}$  levels in the cell are thought to increase to approximately micromolar concentrations on stimulation (5). This concentration would be predicted to fill the high-affinity  $\text{Ca}^{2+}$  binding sites on CRT but not the low-affinity sites. It should be noted, however, that the affinity of CRT for  $\text{Ca}^{2+}$  was measured in vitro. While it is clear that CRT contains at least two classes of binding sites with different affinities for  $\text{Ca}^{2+}$ , demonstrating the  $\text{Ca}^{2+}$  occupancy of particular sites on CRT in the cell may require the development of fluorescence-based assays that can register  $\text{Ca}^{2+}$  binding. This approach could be used to determine if  $\text{Ca}^{2+}$  is constitutively bound to CRT or if  $\text{Ca}^{2+}$  binds reversibly, thereby acting as a regulator of CRT function. We speculate that transient increases in  $\text{Ca}^{2+}$  concentration, theoretically, could promote the assembly of CRT-DBD complexes in the nucleus. The complex could then undergo export to the cytoplasm, where the complex is disassembled, which would be favored by low free  $\text{Ca}^{2+}$  concentrations.

Although the origin of nuclear  $\text{Ca}^{2+}$  remains an issue of debate, it is clear that processes including transcription are regulated by nuclear  $\text{Ca}^{2+}$  (16). Several observations are actually consistent with the notion that  $\text{Ca}^{2+}$  gradients between the nucleus and cytoplasm might be used to promote the assembly and disassembly of CRT complexes. First, the presence of  $\text{Ca}^{2+}$  ATPases in the nuclear envelope and the lack of  $\text{Ca}^{2+}$  buffering and sequestration proteins in the nucleus should prolong the availability of free  $\text{Ca}^{2+}$  (12). Second, the inner membrane of the nuclear envelope contains both major  $\text{Ca}^{2+}$  release channels, which may be activated independently of channels in the ER membrane that face the cytoplasm (36). Third, morphological data show that the ER forms invaginations that reach deep into the nucleus (13), which could place  $\text{Ca}^{2+}$  release channels close to sites of CRT-DBD assembly in the nucleoplasm. While it is clear that  $\text{Ca}^{2+}$  levels in the nucleus do change in response to signaling events, it remains to be established whether  $\text{Ca}^{2+}$  regulation of CRT is a dynamic process that involves reversible binding or whether  $\text{Ca}^{2+}$  is constitutively bound and simply maintains the tertiary structure of CRT.

CRT is thought to function in multiple pathways in the cell (23). In the ER, CRT may function as (i) a chaperone that mediates folding by transiently binding to oligosaccharide side chains and peptide domains, (ii) a  $\text{Ca}^{2+}$  storage protein involved in  $\text{Ca}^{2+}$  homeostasis, and (iii) a factor that directly regulates the activity of the  $\text{Ca}^{2+}$  pumps. At the plasma membrane, CRT may modulate (i) cell migration by interacting with adhesion plaque proteins and (ii) cell-cell interactions by interacting with cell surface proteins. In the nucleocytoplasmic compartment, CRT can regulate (i) the activity of steroid receptors through direct binding to the DBD of these proteins and (ii) the redistribution of steroid receptors to the cytoplasm. Because most of these functions mentioned above have been linked to the  $\text{Ca}^{2+}$  binding activity of CRT, conditions that influence the  $\text{Ca}^{2+}$  levels are predicted to regulate the activity of CRT. In this regard, our findings that  $\text{Ca}^{2+}$  is necessary for the nuclear export function of CRT in vitro and that  $\text{Ca}^{2+}$  depletion results in GR export defects in vivo suggest that this



pathway is used to regulate the nucleocytoplasmic distribution of GR and possibly other steroid hormone receptors. This link between  $\text{Ca}^{2+}$  and GR export could negatively regulate transcription through a nuclear transport pathway, perhaps using  $\text{Ca}^{2+}$  as a second messenger.

#### ACKNOWLEDGMENTS

We thank Gordon Hager, Dona Love, and John Hanover for cell lines and Ian Macara, Dirk Görlich, and Larry Gerace for plasmids.

Financial support was provided by the NIH (GM58639-01 to B.M.P.) and the DOD (DAMD 17-00-1-0048 to F.R.).

#### REFERENCES

- Askjaer, P., T. H. Jensen, J. Nilsson, L. Englmeyer, and J. Kjems. 1998. The specificity of the CRM1-Rev nuclear export signal interaction is mediated by RanGTP. *J. Biol. Chem.* 273:33414-33422.
- Baksh, S., and M. Michalak. 1991. Expression of calreticulin in *Escherichia coli* and identification of its  $\text{Ca}^{2+}$  binding domains. *J. Biol. Chem.* 266:21458-21465.
- Black, B. E., J. M. Holaska, F. Rastinejad, and B. M. Paschal. 2001. DNA binding domains in diverse nuclear receptors function as nuclear export signals. *Curr. Biol.* 11:1749-1758.
- Black, B. E., L. Lévesque, J. M. Holaska, T. C. Wood, and B. M. Paschal. 1999. Identification of an NTF2-related factor that binds Ran-GTP and regulates nuclear protein export. *Mol. Cell. Biol.* 19:8616-8624.
- Brini, M., and E. Carafoli. 2000. Calcium signalling: a historical account, recent developments and future perspectives. *Cell Mol. Life Sci.* 57:354-370.
- Burns, K., B. Duggan, E. A. Atkinson, K. S. Famulski, M. Nemer, R. C. Bleackley, and M. Michalak. 1994. Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. *Nature* 367:476-480.
- Corbett, E. F., K. M. Michalak, K. Oikawa, S. Johnson, L. D. Campbell, P. Eggleton, C. Kay, and M. Michalak. 2000. The conformation of calreticulin is influenced by the endoplasmic reticulum luminal environment. *J. Biol. Chem.* 275:27177-27185.
- Dedhar, S., P. S. Rennie, M. Shago, C. Y. Hagesteijn, H. Yang, J. Filmus, R. G. Hawley, N. Bruchovsky, H. Cheng, R. J. Matusik, and V. Giguere. 1994. Inhibition of nuclear hormone receptor activity by calreticulin. *Nature* 367:480-483.
- Ellgaard, L., R. Riek, T. Herrmann, P. Peter Güttert, D. Braun, A. Helenius, and K. Wüthrich. 2001. NMR structure of the calreticulin P-domain. *Proc. Natl. Acad. Sci. USA* 98:3133-3138.
- Fischer, U., J. Huber, W. C. Boelens, I. W. Mattaj, and R. Luhrmann. 1995. The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell* 82:475-483.
- Fornerod, M., M. Ohno, M. Yoshida, and I. W. Mattaj. 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* 90:1051-1060.
- Fox, J. L., A. D. Burgstahler, and M. H. Nathanson. 1997. Mechanism of long-range calcium signalling in the nucleus of isolated rat hepatocytes. *Biochem. J.* 326:491-495.
- Fricker, M., M. Hollinshead, N. White, and D. Vaux. 1997. Interphase nuclei of many mammalian cell types contain deep, dynamic, tubular membrane-bound invaginations of the nuclear envelope. *J. Cell Biol.* 136:531-544.
- Görlich, D., and U. Kutay. 1999. Transport between the cell nucleus and the cytoplasm. *Annu. Rev. Cell Dev. Biol.* 15:607-660.
- Greber, U. F., and L. Gerace. 1995. Depletion of calcium from the lumen of endoplasmic reticulum reversibly inhibits passive diffusion and signal-mediated transport into the nucleus. *J. Cell Biol.* 128:5-14.
- Hardingham, G. E., S. Chawla, C. M. Johnson, and H. Bading. 1997. Distinct functions of nuclear and cytoplasmic calcium in the control of gene expression. *Nature* 385:260-265.
- Helenius, A., T. S. Trombetta, D. N. Hebert, and J. F. Simons. 1997. Calnexin, calreticulin, and the folding of glycoproteins. *Trends Cell Biol.* 7:193-200.
- Holaska, J. M., B. E. Black, D. C. Love, J. A. Hanover, J. Leszyk, and B. M. Paschal. 2001. Calreticulin is a receptor for nuclear export. *J. Cell Biol.* 152:127-140.
- Holaska, J. M., and B. M. Paschal. 1998. A cytosolic activity distinct from CRM1 mediates nuclear export of protein kinase inhibitor in permeabilized cells. *Proc. Natl. Acad. Sci. USA* 95:14739-14744.
- Johnson, S., M. Michalak, M. Opas, and P. Eggleton. 2001. The ins and outs of calreticulin: from the ER lumen to the extracellular space. *Trends Cell Biol.* 11:122-129.
- Katagiri, Y., K. Takeda, Z. X. Yu, V. J. Ferrans, K. Ozato, and G. Guroff. 2000. Modulation of retinoid signalling through NGF-induced nuclear export of NGFI-B. *Nat. Cell Biol.* 2:435-440.
- Kehlenbach, R. H., A. Dickmanns, A. Kehlenbach, T. Guan, and L. Gerace. 1999. A role for RanBP1 in the release of CRM1 from the nuclear pore complex in a terminal step of nuclear export. *J. Cell Biol.* 145:645-657.
- Krause, K. H., and M. Michalek. 1997. Calreticulin. *Cell* 88:439-443.
- Lindsay, M. E., J. M. Holaska, K. Welch, B. M. Paschal, and I. G. Macara. 2001. Ran-binding protein 3 is a cofactor for Crm1-mediated nuclear protein export. *J. Cell Biol.* 153:1391-402.
- Liu, J., and D. B. DeFranco. 2000. Prolonged nuclear export of glucocorticoid receptor limits its turnover and does not require the exportin 1/CRM1-directed nuclear export pathway. *Mol. Endocrinol.* 14:40-51.
- Love, D. C., T. D. Sweitzer, and J. A. Hanover. 1998. Reconstitution Of HIV-1 Rev nuclear export-independent requirements for nuclear import and export. *Proc. Natl. Acad. Sci. USA* 95:10608-10613.
- McNally, J. G., W. G. Muller, D. Walker, R. Wolford, and G. L. Hager. 2000. The glucocorticoid receptor: rapid exchange with regulatory sites in living cells. *Science* 287:1262-1265.
- Nakiely, S., and G. Dreyfuss. 1999. Transport of proteins and RNAs in and out of the nucleus. *Cell* 99:677-690.
- Ostwald, T. J., and D. H. MacLennan. 1974. Isolation of a high affinity calcium-binding protein from sarcoplasmic reticulum. *J. Biol. Chem.* 249:974-979.
- Paschal, B. M., and L. Gerace. 1995. Identification of NTF2, a cytosolic factor for nuclear import that interacts with nuclear pore complex protein p62. *J. Cell Biol.* 129:925-937.
- Picard, D., and K. R. Yamamoto. 1987. Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *EMBO J.* 6:3333-3340.
- Rastinejad, F. 2001. Retinoid X receptor and its partners in the nuclear receptor family. *Curr. Opin. Struct. Biol.* 11:33-38.
- Rojiani, M. V., B. B. Finlay, V. Gray, and S. Dedhar. 1991. In vitro interaction of a polypeptide homologous to human Ro/SS-A antigen (calreticulin) with a highly conserved amino acid sequence in the cytoplasmic domain of integrin alpha subunits. *Biochemistry* 30:9859-9866.
- Rubio, I., P. Buckle, H. Trutnau, and R. Wetzker. 1997. Real-time assay of the interaction of a GST fusion protein with a protein ligate using resonant mirror technique. *Bio/technology* 22:269-271.
- Saito, Y., Y. Ihara, M. R. Leach, M. F. Cohen-Doyle, and D. B. Williams. 1999. Calreticulin functions in vitro as a molecular chaperone for both glycosylated and non-glycosylated proteins. *EMBO J.* 18:6718-6729.
- Santella, L., and K. Kozuka. 1997. Effects of 1-methyladenine on nuclear calcium transients and meiosis resumption in starfish oocytes are mimicked by the nuclear injection of inositol 1,4,5-triphosphate and cADP-ribose. *Cell Calcium* 22:11-20.
- Schrag, J. D., J. J. Bergeron, Y. Li, S. Borisova, M. Hahn, D. Y. Thomas, and M. Cygler. 2001. The structure of calnexin, an ER chaperone involved in quality control of protein folding. *Mol. Cell* 8:633-644.
- Singh, N. K., C. D. Atreya, and H. L. Nakhasi. 1994. Identification of calreticulin as a rubella virus RNA binding protein. *Proc. Natl. Acad. Sci. USA* 91:12770-12774.
- Stade, K., C. S. Ford, C. Guthrie, and K. Weis. 1997. Exportin 1 (Crm1p) is an essential nuclear export factor. *Cell* 90:1041-1050.
- Steggerda, S. M., and B. M. Paschal. 2002. Regulation of nuclear import and export by the GTPase Ran. *Int. Rev. Cytol.* 217:41-91.
- Stoffler, D., K. N. Goldie, B. Feja, and U. Aebi. 1999. Calcium-mediated structural changes of native nuclear pore complexes monitored by time-lapse atomic force microscopy. *J. Mol. Biol.* 287:741-752.
- Strubing, C., and D. E. Clapham. 1999. Active nuclear import and export is independent of luminal  $\text{Ca}^{2+}$  stores in intact mammalian cells. *J. Gen. Physiol.* 113:239-248.
- Vassilakos, A., M. Michalak, M. A. Lehrman, and D. B. Williams. 1998. Oligosaccharide binding characteristics of the molecular chaperones calnexin and calreticulin. *Biochemistry* 37:3480-3490.
- Wen, W., J. L. Meinkoth, R. Y. Tsien, and S. S. Taylor. 1995. Identification of a signal for rapid export of proteins from the nucleus. *Cell* 82:463-473.
- Wente, S. R. 2000. Gatekeepers of the nucleus. *Science* 288:1374-1377.
- Wiehens, N., and F. Fagotto. 2001. CRM1- and Ran-independent nuclear export of beta-catenin. *Curr. Biol.* 11:18-27.